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14. ABSTRACT

Our objective was to screen libraries of several thousand clinically approved drugs for their ability to suppress the in vivo phenotypes observed in both worm and fish models upon expression of mutant TDP-43 related to ALS. Our hypothesis is that chemical modifiers of TDP-43 in vivo function will provide new therapeutic approaches to ALS. The screen set-up (Aim 1) began immediately for worms and fish during the first 6 months. This consisted of developing video microscopy screens for motility. The first chemical screen (3,500 FDA-approved compounds, Aim 2) began immediately afterwards in worms and was completed during the second half of the year. As hits were identified, the 20 active compounds were rescreened in worms and fish. Most of the active compounds were neuroleptics with the most potent (acting at <1μM) being pimozide. This work was done by PIs Drapeau, Kabashi and Parker.

15. SUBJECT TERMS
TDP-43, C. elegans, zebrafish, motility screens, chemical libraries

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Introduction

Our project is to screen libraries of several thousand small chemicals, including clinically approved drugs, for their ability to suppress the pathogenic phenotypes in three unique *in vivo* genetic models of ALS that we have recently generated. Our new, functionally validated models are worms (*C. elegans*)[1,2], zebrafish (*D. rerio*)[3],[4] and mice (*M. musculus*)[5] expressing ALS-related mutations of the human *TARDBP* gene coding for TDP-43, a recently discovered major contributing factor in ALS. We hypothesize that chemical genetic modifiers of TDP-43 *in vivo* function will provide new therapeutic approaches to ALS[6,7].

During year 1, we had the following specific aims according to our SOW:

- Set-up **motility assays** for TDP-43 worms and zebrafish embryos.
  - Measurement of spontaneous activity in TDP-43 worms.
  - Measurement of the motor response in TDP-43 zebrafish.

- **Screen** TDP-43 worms and zebrafish for restoration of motility with 3k+ FDA-approved compounds.

- **Rescreen** TDP-43 worms and zebrafish for specificity and potency of active compounds.

This work was done by PIs Drapeau, Kabashi and Parker as it concerned only the C. elegans and zebrafish models in the first year.

Body

Study design.
Our department operates a platform for integrated robotic screening of libraries of small chemicals, including 3,500 clinically approved compounds in the Biomol Natural Products, Microsource Discovery Spectrum, Prestwick Commercial Products and Sigma Lopac sets. These were our first screening priority (year 1, including a six month initial set-up period) as validated modifiers ('hits') could be immediately considered for clinical testing. We screened worms and fish by video microscopy for the effects of these compounds on recovery of motility or survival and re-tested hits for dose and time dependence.

Progress.
Motility assays. The screen set-up began immediately for worms and fish during the first 6 months. This consisted of developing video microscopy screens for motility. Previous screens with worms took two weeks to perform as that is the period it takes for them to develop adult-onset phenotypes such as for TDP-43 when grown on a solid substrate. We found a similar result for worms expressing human TDP-43 (see appendix 1[1], Figure 3) from a motoneuron promoter (see appendix 1[1], Figure 1). Further we found that by growing the worms in liquid suspension this accelerated the onset of the phenotype such that by 6 hours it was apparent (see appendix 1[1], Figure 10). This revolutionized our screening protocol as it permits screening within the same working day and makes it possible for one person to screen up to 1,000 molecules per week.

Our stable transgenic zebrafish line required us to raise several generations during the first year before obtaining stable expression and is now ready for use in large scale screening. During the first year we used our established transient model[4,8] to retest hits from the worm screen (i.e. in series rather than in parallel).

Screening. In preliminary tests we had found that methylene blue was effective in protecting against the TDP-43 phenotype in both worms (see appendix 2[2], Figure 1B&2A) and fish (see appendix 2[2], Figure 3) by protecting against cellular stress (see appendix 2[2], Figure 6). This compound was used as a positive control for setting up the larger screens.

The first chemical screen of 3,500 FDA-approved compounds began immediately afterwards in worms and was completed during the second half of the year. 21 hits were identified but 8 were excluded as being irrelevant (insecticides, tumour drugs – in blue in table 1). The other 13 were retested and confirmed in zebrafish. The confirmed hits (in black) are indicated in table 1. Surprisingly, almost all of the confirmed hits were neuroleptics.

Table 1. Summary of FDA-approved compounds with hits in C. elegans, with those confirmed in zebrafish indicated in black.

Source plate	Pick plate	Source well	Name / Action	
CCC0327CP1	P00017066	B010	Mianserine hydrochloride	antidepressant/dopamine
CCC0327CP1	P00017066	C003	Amoxapine	antidep/tetracyclic/noradrenaline reuptake block
CCC0327CP1	P00017066	C004	Cyproheptadine hydrochloride	antihistamine/5HT rec antag
CCC0327CP1	P00017066	G008	Nicergoline	senile dementia/vascular/alpha-1A adrenergic receptor antagonist
CCC0328CP1	P00017067	E008	Kawain	anticonvulsant/anxiolytic/many systems
CCC0329CP1	P00017068	F005	Pimethixene maleate	antihsitamine/anxiety
CCC0329CP1	P00017068	G009	Pimozide	antipsychotic/Tourette/D2
CCC0330CP1	P00017069	B011	Flupentixol dihydrochloride cis-(Z)	antipsychotic/schizo/antidep/Da, 5-HT
CCC0330CP1	P00017069	C009	Chlorprothixene hydrochloride	antipsychotic/5HT, DA
CCC0330CP1	P00017069	C011	Clozapine	antipsychotic/5HT, DA

CCC0330CP1	P00017069	F006	Methiothepin maleate	antipsychotic/5HT, DA
CCC0346CP1	P00017085	G008	Chlorprothixene hydrochloride	antipsychotic/5HT, DA
CCC0352CP1	P00017091	E006	(±)-Octoclothepin maleate	neuroleptic/many systems
		H007	Ivermectin (Insecticide)	Glu (Cl)/GABAR
		F009	Fenbufen	inflammation/cylooxygenase, prostaglandins
		A003	Flavoxate hydrochloride	anticholinergic/bladder
		F009	Melatonin	diurnal/mito, DNA protection/natural!
		F010	Menadione	Vit K/cancer/EGFR
		B011	Avermectin B1 (Insecticide)	insectiside, antihelmintic
		C011	HYPERICIN	antibiotic/kinases/St John wart/DA B-hydroxylase
		D007	DEOXYPHORBOL	antitumour
		D011	MEZEREIN	antitumour/phorbol
		F003	GENISTEIN	antihelmintic/natural!

Rescreening. As hits were identified, the 13 active compounds were rescreened in worms and fish at concentrations ranging from 0.1-100 μM, including control (non-expressing) animals to check for toxicity at higher concentrations. The most potent compound was pimozide, which was active at <1μM in both models.

Key Research Accomplishments

Project Development:

- Development of a rapid liquid suspension assay for TDP-43 worms.
- Validation of methylene blue as a protective compound in both worms and zebrafish.
- Screening of 3,500 FDA-approved compounds in C. elegans with 21 hits (0.6%), 13 of which were neuroleptics that were retained for further study.
- Validation of the neuroleptics in TDP-43 zebrafish.
- Dose-response evaluation in rescreening of all 13 hits in both models.
- Identification of pimozide as the most potent compound from the screen.

Presentations:

- 7th Symposium on ALS, Fondation André-Delambre, Québec, Qc
- 10th Anniversary of the Barbara Turnbull Award in Spinal Cord Research, Toronto
- 20th International Meeting on ALS/Motor Neuron Diseases, Sydney, AUS
- Israel-Canada Workshop in Medical Neuroscience, Jerusalem, Israel
- Brain Research Centre, UBC, Vancouver, CA
- Session on Clinical Research and Practice, ALS Canada, Toronto, ON
- Session speaker, first joint Canadian Human Genetics Conference and Canadian Genetic Epidemiology and Statistical Genetics Meeting, Niagara-on-the-Lake, ON.
- Neuroscience program, Queen's U., Kingston, ON
- Neuroscience Axis, Research Institute of the Univ. Montreal Hospital Centre, Montréal
- Motor Neuron Center, Columbia University, New York, NY
- Centre de recherche de l'Institut en santé mentale de Québec, Québec
- 8th Annual Symposium on ALS of the Fondation André-Delambre, Quebec

Funding/Patents applied for:

- US Patent Office (pending) 61/563,708 (11-21-2011)
Method for treating Amyotrophic Lateral Sclerosis
Drapeau, P., Parker, AJ, Kabashi, E.

- Canadian Institutes for Health Research
Synaptic targets for therapeutic protection of motor function in a genetic model of ALS
(P Drapeau, 04/13-03/18)

Reportable Outcomes

- Development of a rapid liquid suspension assay for TDP-43 worms.
- Validation of methylene blue as a protective compound in both worms and zebrafish.
- Identification of pimozide as a novel potential therapeutic agent.

Conclusion

Our screen has progressed as expected, with set-up and personnel recruitment accomplished in the first half of year 1 and screening of the FDA-approved compounds completed in C. elegans in the second half of the year. 13 hits were confirmed and are mostly neuroleptics, with the most potent being pimozide. Interestingly, it has been hypothesized that neuroleptics can prevent ALS[10], suggesting that pimozide may be of novel therapeutic potential. If correct this will have validated our strategy.

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PLoS one

Mutant TDP-43 and FUS Cause Age-Dependent Paralysis and Neurodegeneration in *C. elegans*

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Abstract

Mutations in the DNA/RNA binding proteins TDP-43 and FUS are associated with Amyotrophic Lateral Sclerosis and Frontotemporal Lobar Degeneration. Intracellular accumulations of wild type TDP-43 and FUS are observed in a growing number of late-onset diseases suggesting that TDP-43 and FUS proteinopathies may contribute to multiple neurodegenerative diseases. To better understand the mechanisms of TDP-43 and FUS toxicity we have created transgenic *Caenorhabditis elegans* strains that express full-length, untagged human TDP-43 and FUS in the worm's GABAergic motor neurons. Transgenic worms expressing mutant TDP-43 and FUS display adult-onset, age-dependent loss of motility, progressive paralysis and neuronal degeneration that is distinct from wild type alleles. Additionally, mutant TDP-43 and FUS proteins are highly insoluble while wild type proteins remain soluble suggesting that protein misfolding may contribute to toxicity. Populations of mutant TDP-43 and FUS transgenics grown on solid media become paralyzed over 7 to 12 days. We have developed a liquid culture assay where the paralysis phenotype evolves over several hours. We introduce *C. elegans* transgenics for mutant TDP-43 and FUS motor neuron toxicity that may be used for rapid genetic and pharmacological suppressor screening.

Citation: Vaccaro A, Tauffenberger A, Aggad D, Rouleau G, Drapeau P, et al. (2012) Mutant TDP-43 and FUS Cause Age-Dependent Paralysis and Neurodegeneration in *C. elegans*. PLoS ONE 7(2): e31321. doi:10.1371/journal.pone.0031321

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Introduction

Amyotrophic Lateral Sclerosis (ALS) is a late-onset progressive disease affecting motor neurons ultimately causing fatal paralysis [1,2]. Most cases are sporadic, but ~10% of patients have an inherited familial form of the disease. Dominant mutations in SOD1 (copper/zinc superoxide dismutase 1) account for ~20% of familial ALS cases and ~1% of sporadic cases [1]. The recent discovery of mutations in TAR DNA-binding protein-43 (TDP-43) and Fused in sarcoma (FUS, also named TLS) in both familial ALS and frontotemporal dementia (FTD) has shifted research into disease mechanisms and potential therapeutics [3–9].

TDP-43 and FUS are evolutionarily conserved DNA/RNA binding proteins that shuttle between the nucleus and cytoplasm having multiple roles including DNA transcription and RNA processing [3,9–12]. Mutant TDP-43 and FUS (mTDP-43 and mFUS) are found in cytoplasmic inclusions in the disease state while the accumulation of wild type TDP-43 and FUS (wtTDP-43 and wtFUS) are observed in an increasing number of disorders including Alzheimer's Disease, Parkinson's Disease and the polyglutamine diseases (reviewed in [10]). The pathogenic mechanisms for mutant TDP-43 and FUS age-dependent neuronal toxicity remain unclear. As of now there is no consensus whether mutant TDP-43 and FUS employ a loss-of-function, a gain-of-function, or both in motor neuron cell death.

Since TDP-43 and FUS are evolutionarily conserved we used the nematode *Caenorhabditis elegans* to investigate mutant TDP-43 and FUS age-dependent neurodegeneration. We created transgenic nematodes that express full-length wild type or mutant TDP-43 and FUS in the worm's GABAergic motor neurons. Transgenic TDP-43 and FUS worms recapitulate a salient feature of ALS; they display adult-onset, age-dependent, progressive paralysis and degeneration of motor neurons. Importantly, mTDP-43 and mFUS, but not wtTDP-43 and wtFUS, strains show the presence of insoluble proteins in extracts from whole animals suggesting that protein misfolding may be a primary cause of toxicity. We introduce a genetically tractable platform to investigate motor neuron toxicity caused by mutant TDP-43 and FUS that can be used for suppressor screening.

Results

Transgenic worms expressing full-length human TDP-43 or FUS in motor neurons display age-dependent paralysis

Since ALS is a motor neuron disease we expressed wild type and mutant human TDP-43 and FUS proteins in the worm's 26 GABAergic motor neurons with the vesicular GABA transporter (*unc-47*) promoter (Figures 1A, B) [13]. Multiple transgenic strains carrying extrachromosomal arrays were obtained by microinjection and stable lines with chromosomally-integrated transgenes

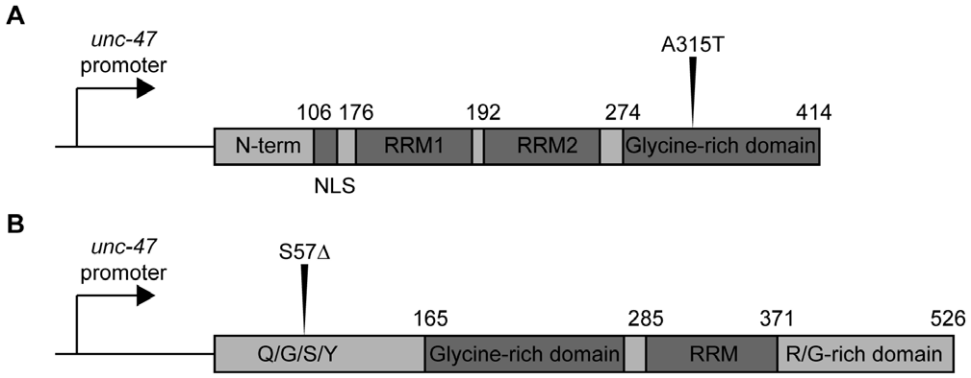

Figure 1. TDP-43 and FUS transgene constructs. (A) Full-length wild type human TDP-43 and the clinical mutation A315T were cloned into a vector for expression in motor neurons by the *unc-47* promoter and injected into *C. elegans*. (B) Full-length wild type human FUS and the clinical mutation S57Δ were cloned into the *unc-47* expression vector and injected into *C. elegans*. RRM (RNA Recognition Motif), Q/G/S/Y (Glutamine-Glycine-Serine-Tyrosine-rich region), R/G (Arginine-Glycine-rich region), NLS (Nuclear localization signal).
doi:10.1371/journal.pone.0031321.g001

were isolated after UV-irradiation [14]. Both wild type TDP-43 and the ALS-associated A315T mutant proteins were expressed in transgenic worms as detected by immunoblotting of worm protein extracts with a human specific TDP-43 antibody (Figure 2A) [4]. Similarly, using a FUS antibody we confirmed the expression of wild type and the ALS-linked S57Δ mutant proteins by western blotting (Figure 2B) [15].

All strains were morphologically normal and showed no adverse phenotypes during development. However, during adulthood the transgenic strains begin to display uncoordinated motility phenotypes that progressed to paralysation. Paralysis was age-dependent and occurred at higher rate for mTDP-43 and mFUS worms compared to wtTDP-43 and wtFUS transgenics (Figures 3 A, B). Typically, after 12–13 days on plates 100% of the mTDP-43 and mFUS worms were paralysed while only 20% of the wtTDP-43 and wtFUS worms were affected. The low rate of paralysis for wtTDP-43 and wtFUS strains is comparable to what is observed in transgenics expressing GFP from the same *unc-47* promoter (Figure 3C). Additionally, the paralysis assay is widely used to study age-dependent degenerative phenotypes and is not observed in wild type non-transgenic worms until they reach advanced age (approximately 20 days) [16–18]. Finally, motility defects and adult onset paralysis have been previously observed in worms with degenerating GABAergic motor neurons suggesting that mTDP-43 and mFUS may negatively affect GABAergic neuronal function and survival [19].

TDP-43 and FUS transgenics have normal lifespans

One of the signs of aging in worms is decreased motility [18,20]. Thus the progressive paralysis phenotypes observed in the TDP-43 and FUS transgenics may be due to overall decreased health from the expression of toxic non-native proteins leading to accelerated mortality, a part of which is a decline in motility. We conducted lifespan analyses and observed that all of the transgenics had lifespans indistinguishable from non-transgenic wild type N2 worms (Figures 4A, B and Table S1). These observations suggest that the paralysis observed in our models is specific to the expression of TDP-43 and FUS in motor neurons and not due to secondary effects from general sickness and reduced lifespan.

TDP-43 and FUS cause neuronal dysfunction

The progressive paralysis phenotypes caused by mTDP-43 and mFUS suggest there may be motor neuron dysfunction and/or

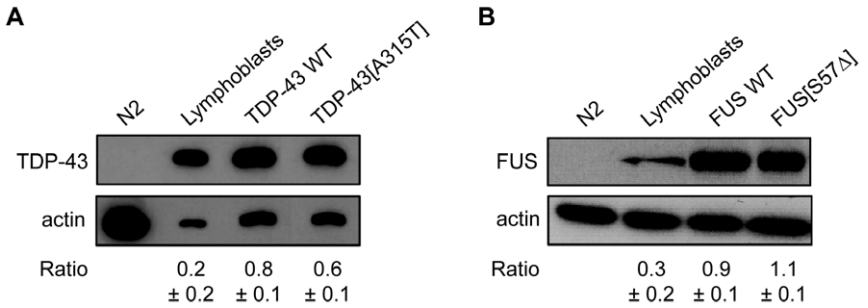

Figure 2. Expression of human TDP-43 and FUS proteins in *C. elegans* transgenics. (A) Total protein levels from non-transgenic worms, human lymphoblast cells and transgenic worms expressing wtTDP-43 or mTDP-43. Staining with a human TDP-43 antibody showed no signal for non-transgenic worms but a signal corresponding to full-length human TDP-43 at ~45 kDa in size was observed in extracts from human cells and the two transgenic TDP-43 worm strains. wtTDP-43 and mTDP-43 strains showed comparable protein expression levels. (B) Total protein levels from non-transgenic worms, human lymphoblast cells and transgenic worms expressing wtFUS or mFUS. Using a human FUS antibody, no signal was detected in non-transgenic worms, but a signal corresponding to full-length human FUS at ~75 kDa in size was observed in extracts from lymphoblast cells and the transgenic FUS worm strains. wtFUS and mFUS worms showed identical levels of protein expression. For all experiments actin staining was used as a loading control and expression ratios ± SEM of TDP-43 or FUS to actin was determined from 3 independent experiments. Representative western blots are shown.
doi:10.1371/journal.pone.0031321.g002

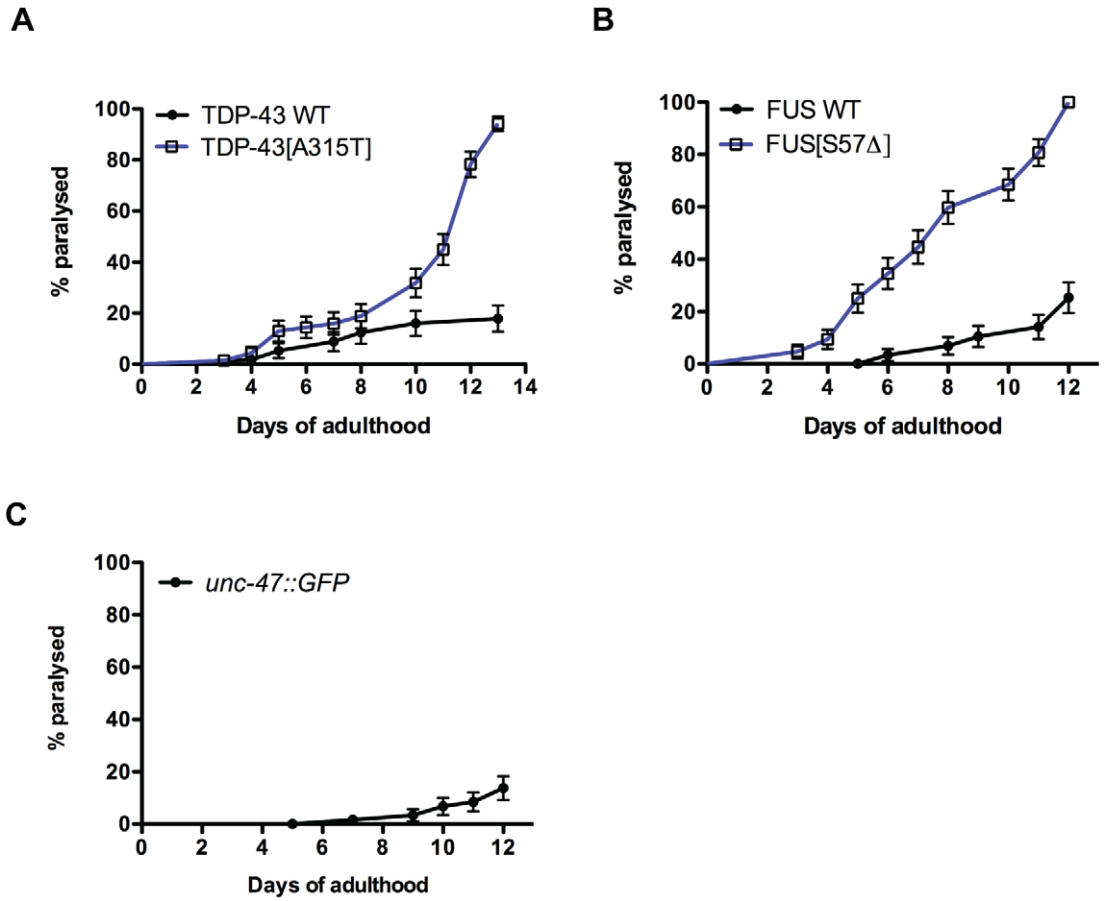

Figure 3. Mutant TDP-43 and FUS cause adult-onset, age-dependent paralysis in *C. elegans*. Transgenics were monitored from the adult stage and scored daily for paralysis. (A) mTDP-43 worms show a rate of progressive paralysis that is greater than transgenics expressing wtTDP-43 ($P<0.001$). (B) Transgenics expressing mFUS become paralysed significantly sooner than wtFUS control transgenics ($P<0.001$). (C) Transgenic worms expressing GFP in motor neurons show low levels of paralysis.
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degeneration in these animals. *C. elegans* body wall muscle cells receive excitatory (acetylcholine) and inhibitory (GABA) inputs to coordinate muscle contraction/relaxation and facilitate movement [21,22]. Body wall muscle activity can be measured indirectly with the acetylcholinesterase inhibitor aldicarb [23]. Exposure to aldicarb causes accumulation of acetylcholine at neuromuscular junctions resulting in hyperactive cholinergic synapses, muscle hypercontraction, and acute paralysis [23]. Hypersensitivity to

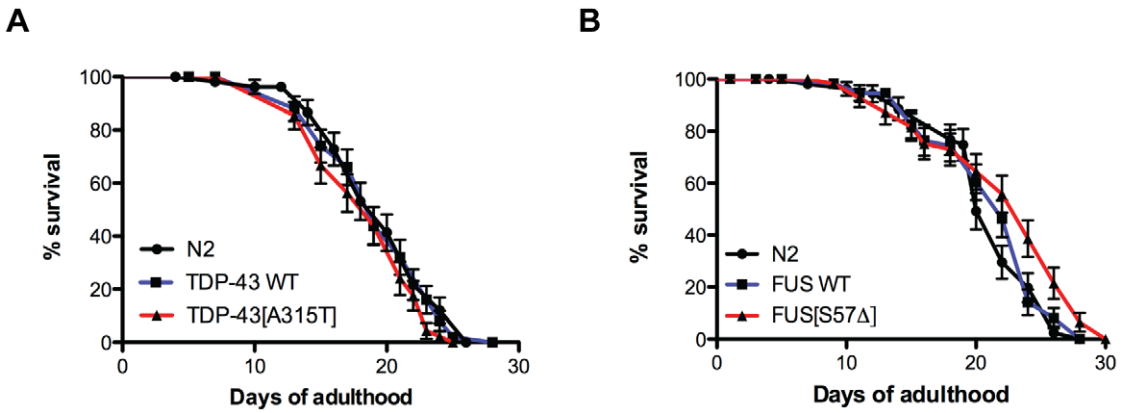

Figure 4. TDP-43 and FUS transgenes do not affect lifespan. Beginning at Day 1 of adulthood we tested the lifespans of wild type non-transgenic N2 worms and transgenics expressing (A) wtTDP-43 and mTDP-43 as well as (B) animals expressing wtFUS and mFUS. Animals expressing TDP-43 or FUS transgenes had lifespans indistinguishable from N2 worms.
doi:10.1371/journal.pone.0031321.g004

aldicarb-induced paralysis has been used to identify genes that increase acetylcholine secretion or decrease inhibitory GABA signalling [24]. For example mutants lacking genes required for GABA transmission like the vesicular GABA transporter *unc-47* are hypersensitive to aldicarb-induced paralysis [25]. To investigate if our TDP-43 and FUS transgenics had abnormal activity at the neuromuscular junction we exposed the animals to aldicarb. We observed that, like *unc-47* mutants, mTDP-43 and mFUS animals were hypersensitive to aldicarb-induced paralysis, while wtTDP-43 and wtFUS transgenics showed a rate paralysis identical to non-transgenic N2 worms (Figures 5A, B). These data suggest that the inhibitory GABA signalling is impaired in mTDP-43 and mFUS transgenics. *unc-47* mutants are classically described as having a "shrinker" phenotype, where in response to touch the worm does not move away but instead the whole body undergoes longitudinal shortening [21], and we observed that the shrinker phenotype was weakly penetrant in adult mTDP-43 and mFUS worms. To determine if impaired GABAergic neurotransmission contributed to the paralysis phenotype we examined two *unc-47* loss-of-function mutants and they both showed age-dependent paralysis, a phenotype not previously reported for *unc-47* (Figure 5C) [21]. Thus, mTDP-43 and mFUS cause neuronal dysfunction in GABA neurons leading to progressive motility defects culminating in paralysis, a phenotype similar to animals deficient in GABAergic signalling.

TDP-43 and FUS cause progressive degeneration of motor neurons

Many neurodegenerative diseases are characterized by neuronal dysfunction prior to degeneration [26]. To investigate if the progressive paralysis phenotypes in our TDP-43 and FUS transgenics were accompanied by neurodegeneration we crossed all of the transgenics with an integrated reporter (*unc-47p::GFP*) that expresses GFP in the same GABAergic motor neurons [13] (Figures 6A, B). Similar to reports from another *C. elegans* TDP-43 toxicity model [27], we observed gaps/breaks in motor neuron processes in TDP-43 and FUS animals compared to animals expressing *unc-47p::GFP* alone (Figures 6 C–F). We extended our analysis by scoring degeneration in living GFP, wtTDP-43, mTDP-43, wtFUS and mFUS transgenics at days 1, 5 and 9 of adulthood. We observed that degeneration was age-dependent and occurred at higher rate for the mTDP-43 and mFUS animals compared to the wtTDP-43 and wtFUS transgenics (Figure 6G). Thus our TDP-43 and FUS transgenics mimic the adult-onset, gradual decline of neuronal function ultimately resulting in age-dependent motor neuron degeneration seen in diseases like ALS.

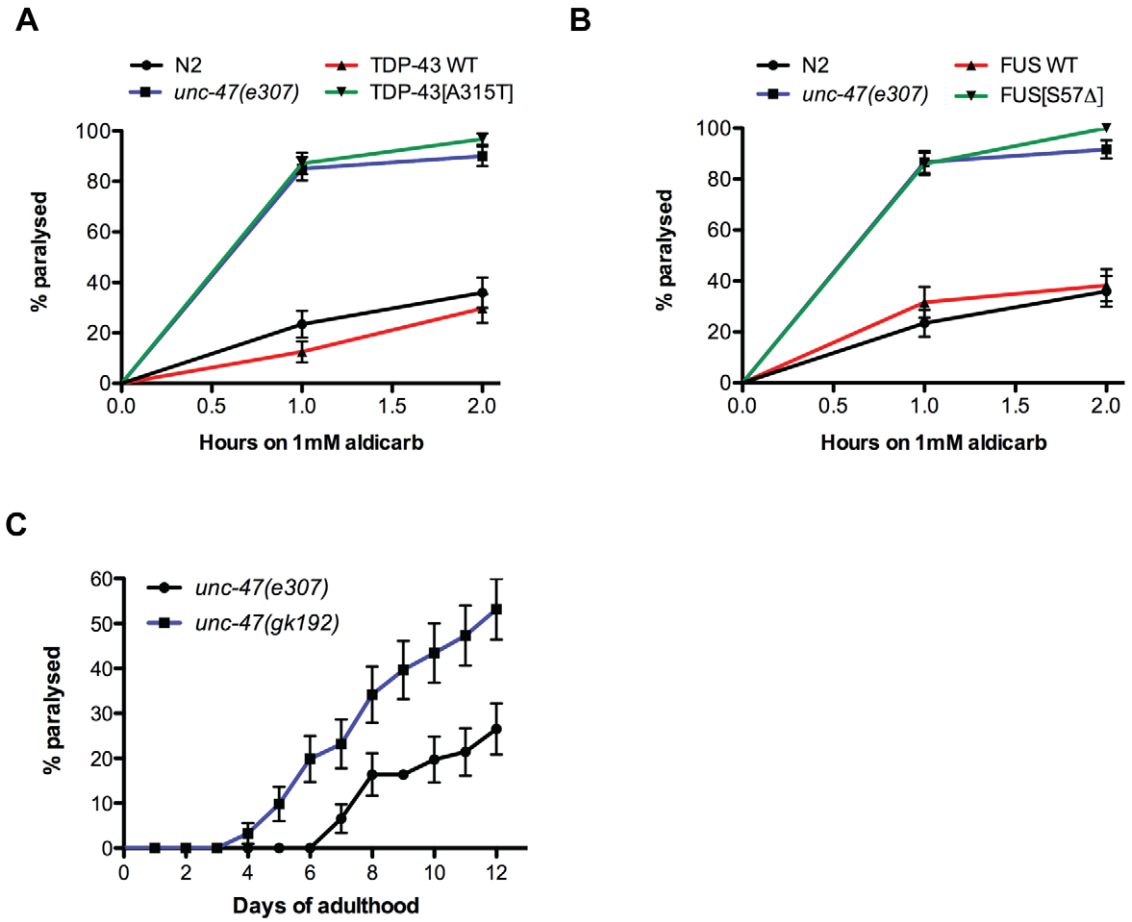

Figure 5. Mutant TDP-43 and FUS impair synaptic transmission. (A) Cholinergic neuronal transmission was measured by determining the onset of paralysis induced by the cholinesterase inhibitor aldicarb. *unc-47(e307)* mutants and mTDP-43 transgenics were hypersensitive to aldicarb-induced paralysis compared to either wtTDP-43 transgenics or N2 worms ($P<0.001$ for *unc-47* or mTDP-43 compared to N2 or wtTDP-43 worms). (B) mFUS transgenics and *unc-47(e307)* mutants were more sensitive to aldicarb induced paralysis compared to either wtFUS transgenics or N2 controls ($P<0.001$). (C) *unc-47* mutants grown on regular worm plates showed age-dependent progressive paralysis.
doi:10.1371/journal.pone.0031321.g005

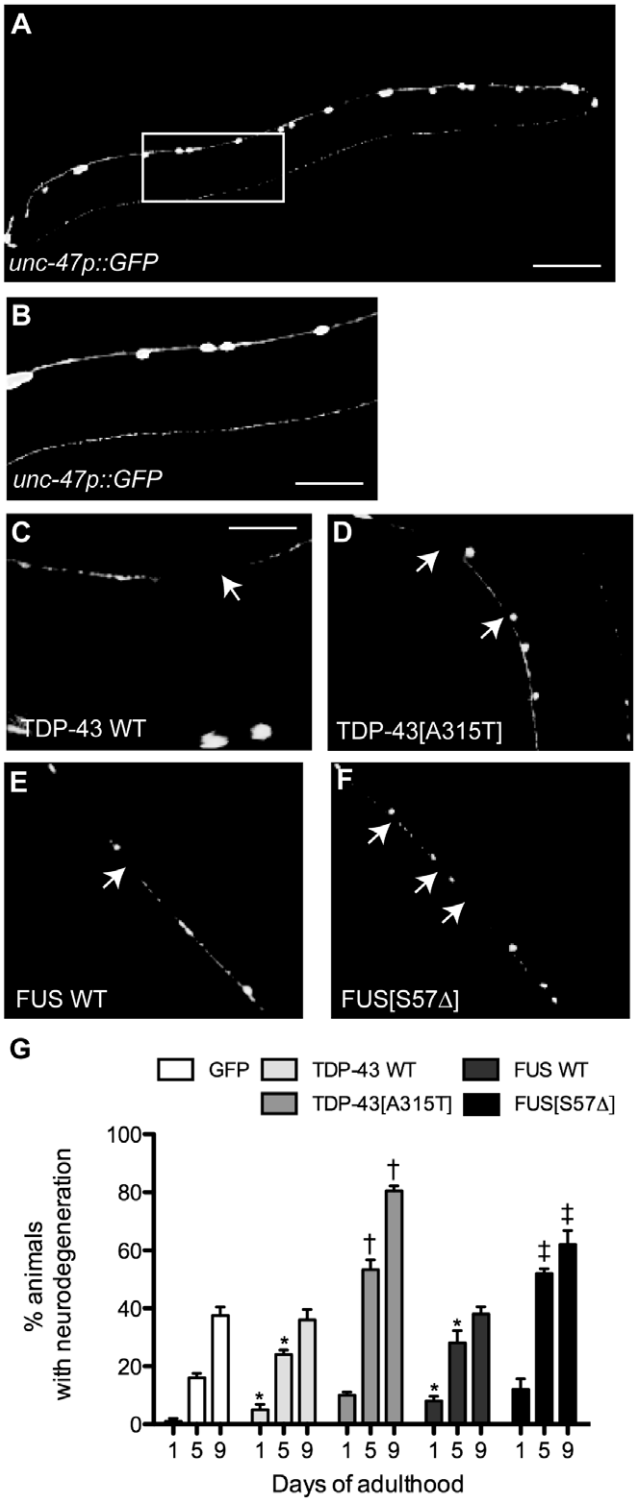

Figure 6. Mutant TDP-43 causes motor neuron degeneration. Shown are representative photos of living, adult *unc-47p::GFP*, *unc-47p::GFP;TDP-43*, and *unc-47p::GFP;FUS* transgenics. (A) Image of an entire *unc-47p::GFP* worm showing the GABAergic motor neurons. Scale bar represents 50 μm. (B) High-magnification of the framed area from (A) showing wild type morphology of motor neurons. Scale bar represents 20 μm. High magnification of motor neurons labelled with *unc-47p::GFP* in (C) wtTDP-43, (D) mTDP-43, (E) wtFUS and (F) mFUS transgenics showing gaps along neuronal processes (arrows). Scale bar represents 10 μm for photos (C) to (F). (G) Quantification of neurodegeneration in transgenic worms at days 1, 5 and 9 of adulthood. * wtTDP-43 and wtFUS have a higher rate of neurodegeneration compared to *unc-47p::GFP* controls at days 1 and 5 of adulthood ($P<0.05$). †mTDP-43 transgenics have a higher rate of neurodegeneration at days 5 and 9 compared to wtTDP-43 transgenics ($P<0.001$). ‡mFUS transgenics show an enhanced rate of neurodegeneration at days 5 and 9 of adulthood in compared to wtFUS transgenics ($P<0.001$).
doi:10.1371/journal.pone.0031321.g006

Mutant TDP-43 and FUS are highly insoluble

Since TDP-43 and FUS are prone to aggregation in several model systems including *C. elegans*, we tested if the same was true for our transgenics [27–33]. To examine if protein misfolding is more pronounced for strains expressing mTDP-43 and mFUS, we used a biochemical assay to detect protein aggregation. Homogenized protein extracts from transgenic worms were separated into supernatant (detergent-soluble) and pellet (detergent-insoluble) fractions [30]. Immunoblotting the TDP-43 transgenics with a human TDP-43 antibody revealed the accumulation of mTDP-43 in the pelleted, insoluble fraction, while wtTDP-43 proteins were predominantly detected in the supernatant, or soluble fractions (Figure 7A). Similar results were obtained for the FUS transgenics where immunoblotting with a human FUS antibody showed that mFUS accumulated in the insoluble pellet fraction while wtFUS proteins remained soluble (Figure 7B). These data suggest that mTDP-43 and mFUS proteins are susceptible to misfolding leading to insolubility and aggregation that may contribute to motor neuron dysfunction and degeneration.

Next focusing on the mTDP-43 and mFUS transgenics we fixed whole *unc-47p::GFP;mTDP-43* and *unc-47p::GFP;mFUS* worms and respectively stained them with human TDP-43 and human FUS antibodies. We detected mTDP-43 and mFUS in both the nuclei and cytoplasm of motor neurons (Figure 8). The cytoplasmic accumulation of mTDP-43 and mFUS in our transgenics is consistent with findings in patients suggesting that these proteins misfold leading to intracellular build-up and aggregation [10].

Finally, we noticed that the fixed mTDP-43 and mFUS showed gaps or breaks along the GFP labelled neuronal processes similar to what was observed in living animals (Figures 6D, F). To confirm that neurodegeneration was not simply due to loss of GFP signals, we stained whole *unc-47p::GFP;mTDP-43* and *unc-47p::GFP;mFUS* worms for GABA [22]. We observed that the gaps along the processes as visualized by a loss of GFP signal likewise corresponded to a loss of GABA staining (Figure 9). Altogether these data suggest that the expression of TDP-43 and FUS lead to degeneration of motor neurons as has been observed for TDP-43 in other worm models [27].

Paralysis phenotypes are enhanced in liquid culture

One goal in developing these transgenics is for use in genetic and pharmacological suppressor screens. TDP-43 and FUS transgenics may have decreased inhibitory GABA signalling ultimately causing muscle hypercontraction leading to paralysis. When grown on solid media the mTDP-43 and mFUS paralysis phenotypes manifest over a period of 5 to 13 days (Figure 3). Worms grown in liquid culture exhibit a stereotypical swimming motion that is considerably more vigorous than worms crawling on solid media [34]. We hypothesized that placing worms in liquid culture would increase activity at the neuromuscular junction and precipitate paralysis phenotypes much earlier than worms grown on solid media.

Using age-synchronized worms we transferred young adult TDP-43 and FUS transgenics to 96-well plates with liquid media and scored their motility every 2 hours. We observed a rapid

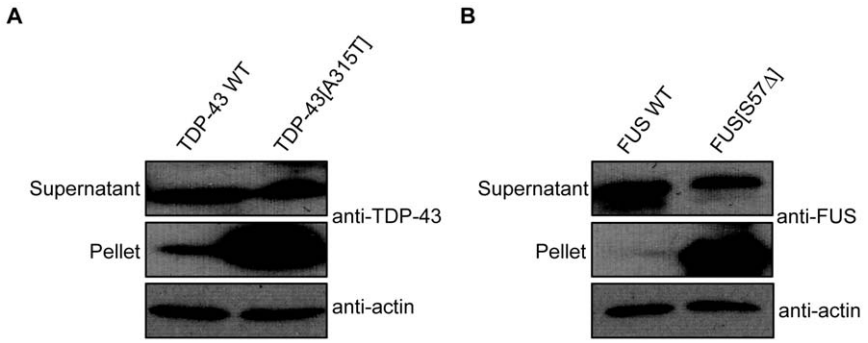

Figure 7. Mutant TDP-43 and FUS are highly insoluble. Shown are representative images from western blotting of the soluble supernatant and insoluble pellet fractions of protein extracts from transgenic TDP-43 and FUS strains. (A) Blotting against TDP-43 shows that a large proportion of the TDP-43 signal resides in the insoluble fraction for mTDP-43 worms, while the signal is largely soluble for the wtTDP-43 samples. (B) Immunoblotting with a human FUS antibody revealed that mFUS proteins primarily resided in the insoluble fractions while wtFUS proteins were exclusively soluble. Immunoblotting for actin was used as the loading control.
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onset of paralysis for the mTDP-43 and mFUS lines with approximately 80% of the population becoming immobile after 6 hours progressing to 100% paralysis after 12 hours (Figure 10A, B Videos S1, S2, S3, S4). wtTDP-43 and wtFUS animals also showed increased paralysis but at a much lower rate, with approximately 20% of the animals immobile after 6 hours moving to 80% paralysis after 12 hours (Figure 10, Videos S5, S6, S7, S8). Non-transgenic N2 animals showed a very low rate of paralysis of approximately 15% after 12 hours (Figure 10C, Videos S9, S10). In comparison, approximately 50% of transgenic *unc-47p::GFP* control animals were paralysed after 12 hours, a rate intermediate between non-transgenic N2 worms and transgenic wtTDP-43 and wtFUS animals (Figure 10C, Videos S11, S12). The difference between wild type and mutant transgenic lines is easy to distinguish, particularly at 6 hours, and suggests that this phenotype may be used for rapid genetic and chemical screening.

Discussion

Here we introduce a novel *C. elegans* platform for investigating mechanisms of motor neuron toxicity caused by mTDP-43 and mFUS. To more closely model human disease we chose to express full-length human TDP-43 and FUS without additional tags since the inclusion of tags like GFP can mask or enhance the phenotypes of wild type and mutant proteins [35,36]. Additionally, we reasoned that restricting expression to a smaller set of neurons might produce phenotypes less severe, or later, than observed in other *C. elegans* models [27,29,30,33]. Since ALS is characterized by degeneration of the motor neurons we engineered strains expressing human TDP-43 and FUS in the animal's 26 GABAergic neurons [13,22]. Additionally, ALS patients show cortical hyperexcitability that may be due to reduced inhibitory signalling from the GABAergic system [37,38]. We believe our transgenic mTDP-43 and mFUS worms recapitulate this patho-

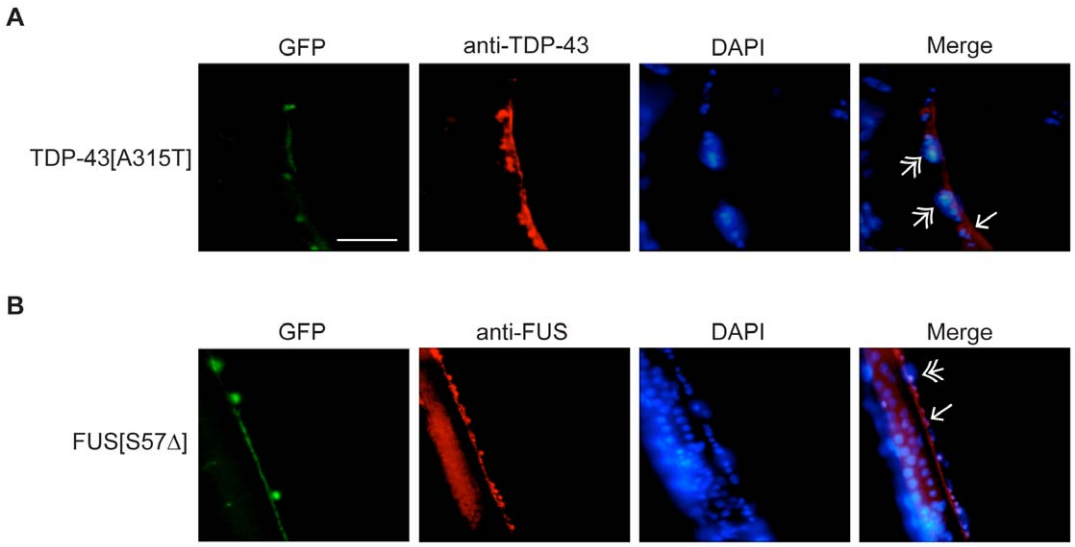

Figure 8. Mutant TDP-43 and FUS aggregate in vivo. (A) Representative image of a fixed *unc-47p::GFP;mTDP-43* worm stained with a human TDP-43 antibody. The green channel shows GFP labelled motor neurons. Antibody staining (red signal) revealed aggregation of TDP-43 signals in motor neurons. Staining of motor neuron nuclei with DAPI (blue signal) revealed that TDP-43 is both cytoplasmic (single arrowhead) and nuclear (double arrowhead). Scale bar represents 10 μm for all photos. (B) Staining of *unc-47p::GFP;mFUS* worms with a human FUS antibody (red signal) and DAPI (blue signal) revealed cytoplasmic (single arrowhead) and nuclear (double arrowhead) accumulations in motor neurons.
doi:10.1371/journal.pone.0031321.g008

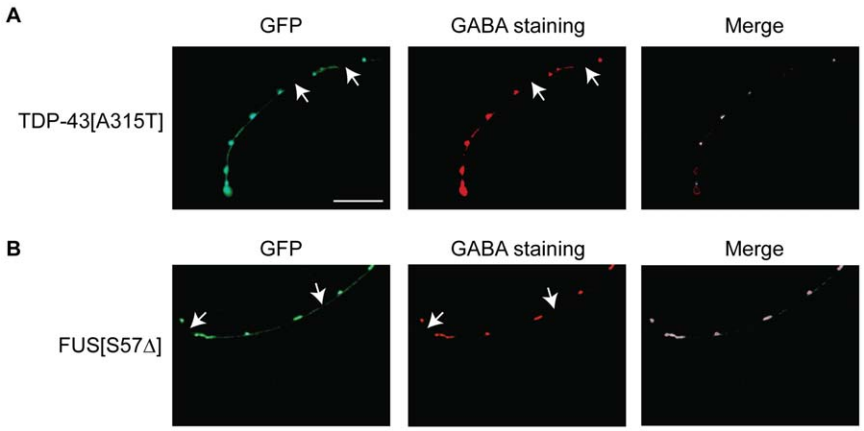

Figure 9. Decreased GABA staining in mutant TDP-43 and FUS worms. (A) Fluorescent micrograph of a fixed *unc-47p::GFP;mTDP-43* worm stained with a GABA antibody revealed neurodegeneration in motor neurons that mirrors the loss of GFP signals. Scale bar represents 10 μm for all photos. (B) Staining of *unc-47p::GFP;mFUS* worms also showed loss of GABA signals similar to the loss of GFP in the motor neurons.
doi:10.1371/journal.pone.0031321.g009

physiological mechanism; they show decreased GABA staining and are hypersensitive to the acetylcholinesterase inhibitor aldicarb, suggesting a reduction of inhibitory GABA input at neuromuscular junctions [24,25]. In our models sensitivity to aldicarb can be detected in day 1 adult worms, while paralysis and motor neuron degeneration can first be detected starting at day 5 of adulthood demonstrating that similar to ALS, neuronal dysfunction occurs prior to neurodegeneration [39].

Importantly, our transgenic TDP-43 and FUS animals only begin to show motility defects once they have reached adulthood a feature absent from other models [27,29,30,33]. Thus our models mirror a prominent clinical feature of ALS, they display adult-

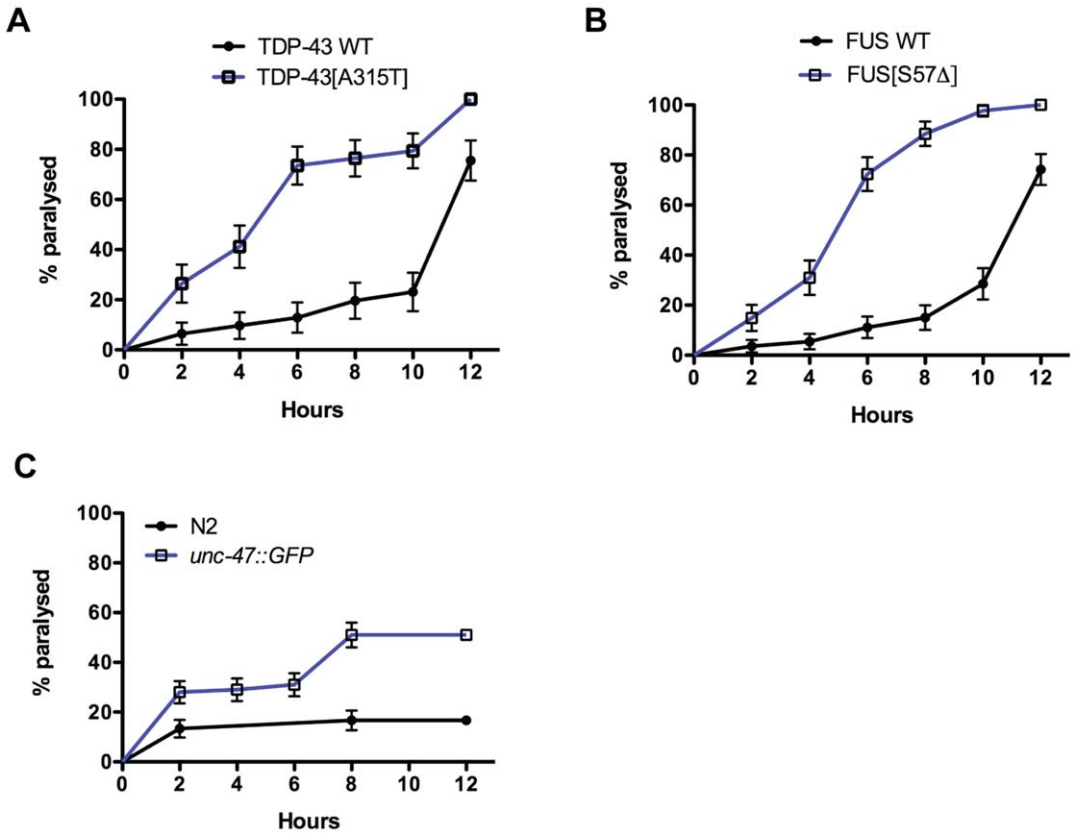

Figure 10. Accelerated paralysis phenotypes for TDP-43 and FUS transgenics in liquid culture. (A) Paralysis phenotypes resolve over a number of hours for wtTDP-43 and mTDP-43 worms grown in liquid culture. mTDP-43 worms have a faster rate of paralysis compared to wtTDP-43 transgenics ($P<0.001$). (B) Transgenic mFUS worms show motility defects and become paralysed at a rate faster than wtFUS controls ($P<0.001$). (C) *unc-47p::GFP* transgenics have an increased rate of paralysis compared to non-transgenic N2 worms ($P<0.001$).
doi:10.1371/journal.pone.0031321.g010

onset, age-dependent, progressive paralysis [40,41]. Additionally, unlike previously described TDP-43 and FUS models based on pan-neuronal expression [27,30,33] our transgenics do not show reduced lifespan suggesting the behavioural phenotypes observed in our transgenics are not influenced by general sickness. Our transgenics do share many features with other neuronal-based models, notably the aggregation and insolubility of mutant TDP-43 and FUS as well as degeneration of motor neurons suggesting there may be common mechanisms of toxicity amongst the models [27,29,30,32,33,42–45]. However, cytoplasmic aggregation of TDP-43 and FUS is a prominent feature of the human pathologies and this is seen in a recently described worm FUS model [33], but is absent from previously reported TDP-43 models [27,29,30]. We detect TDP-43 and FUS in both the nucleus and the cytoplasm of motor neurons from young adult (Day 1) transgenics. The preferential toxicity of mutant TDP-43 and FUS alleles along with their cytoplasmic accumulation suggests our models may recapitulate aspects of neurotoxicity relevant to the disease state.

With no clear mechanism for TDP-43 and FUS neuronal toxicity it is currently not possible to design *in vitro* assays for high-throughput drug screening. Thus the further development and characterization of *in vivo* models for neurodegeneration will guide studies in mammalian systems. We believe our models strike an optimal balance between strong, age-dependent phenotypes and the expression of mutant proteins in relatively few neurons and may be useful for modifier screening. In terms of sensitivity, genetic mechanisms and/or small molecules need only to work on 26 neurons to achieve suppression. In terms of speed, our transgenics offer the possibility of medium-throughput suppressor screening based on the accelerated paralysis phenotype of mTDP-43 and mFUS worms grown in liquid culture. mTDP-43 and mFUS cause neuronal dysfunction in advance of motor neuron degeneration. The path from protein misfolding to neuronal dysfunction and cell death takes many decades in humans and it may be more efficient to target therapies to early pathogenic stages. Thus using simple systems to screen for suppression of neuronal dysfunction may be useful to prevent subsequent neurodegeneration.

A number of models for TDP-43 and FUS toxicity in various systems have been described, but there is still no clear answer whether TDP-43 and FUS neuronal toxicity are due to a loss/gain of function of these proteins individually or together in some common genetic pathway [44–46]. Furthermore it is still unclear if all TDP-43 and FUS mutations share similar pathogenic mechanisms but having similarly constructed models for each may address this question. Now that we have validated the *unc-47* motor neuron approach for modelling toxicity, future work will focus on the development of new transgenics with additional TDP-43 and FUS mutations.

We present here novel transgenics for investigating age-dependent motor neuron toxicity caused by mutant TDP-43 and FUS. We expect these strains will be useful for identifying genetic and chemical suppressors to give insights into disease mechanisms and support the development of new therapies for age-dependent neurodegeneration.

Materials and Methods

Nematode strains

Standard methods of culturing and handling worms were used [47]. Worms were maintained on standard NGM plates streaked with OP50 *E. coli*. Strains used in this study were obtained from the *C. elegans* Genetics Center (University of Minnesota, Minneapolis) and include: N2, *oxIs12[unc-47p::GFP+lin-15]*, *unc-47(e307)*, *unc-47(gk192)* and *unc-119(ed3)*.

Transgenic TDP-43 and FUS worms

Human cDNAs for wild type and mutant TDP-43[A315T], and wild type and mutant FUS-TLS[S57Δ] were obtained from Dr. Guy Rouleau (CRCHUM, Université de Montréal). The cDNAs were amplified by PCR and cloned into the Gateway vector pDONR221 following the manufacturer's protocol (Invitrogen). Multisite Gateway recombination was performed with the pDONR TDP-43 and FUS clones along with clones containing the *unc-47* promoter (kind gift from Dr. Erik Jorgensen, University of Utah), the *unc-54* 3′UTR plasmid pCM5.37 (Dr. Geraldine Seydoux, Johns Hopkins, Addgene plasmid 17253) and the destination vector pCFJ150 to create *unc-47*::TDP-43 and *unc-47*::FUS expression vectors. Transgenic lines were created by microinjection of *unc-119(ed3)* worms, multiple lines were generated and strains behaving similarly were kept for further analysis. Transgenes were integrated by UV irradiation and lines were outcrossed to wild type N2 worms 5 times before use. The main strains used in this study include: *xqIs132[unc-47::TDP-43-WT;unc-119(+)]*, *xqIs133[unc-47::TDP-43[A315T];unc-119(+)]*, *xqIs173[unc-47::FUS-WT;unc-119(+)]*, and *xqIs98[unc-47::FUS[S57-Δ];unc-119(+)]*.

Paralysis assays on plates

For worms expressing TDP-43 or FUS, 20–30 adult day 1 animals were picked to NGM plates and scored daily for movement. Animals were counted as paralyzed if they failed to move upon prodding with a worm pick. Worms were scored as dead if they failed to move their head after being prodded in the nose and showed no pharyngeal pumping. All experiments were conducted at 20°C.

Lifespan assays

Worms were grown on NGM-FUDR plates to prevent progeny from hatching. 20 animals/plate by triplicates were tested at 20°C from adult day 1 until death. Worms were declared dead if they did not respond to tactile or heat stimulus. Survival curves were produced and compared using the Log-rank (Mantel-Cox) test.

Aldicarb test

To evaluate synaptic transmission, worms were grown on NGM and transferred to NGM plates +1 mM aldicarb at adult day 1. Paralysis was scored after 1 and 2 hours on aldicarb plates. Animals were counted as paralyzed if they failed to move upon prodding with a worm pick. All tests were performed at 20°C.

Liquid culture protocol

Synchronized populations of worms were obtained by hypochlorite extraction. Young adult worms were distributed in 96-wells plate (20 µl per well; 20–30 worms per well), containing DMSO or test compounds and incubated for up to 6 h at 20°C on a shaker. The motility test was assessed by stereomicroscopy. Videos of worms were taken with on an Olympus S7x7 stereomicroscope equipped with a Grasshopper GRAS-03K2M camera using Flycap software (Point Grey Research) at a rate of 300 frames per second.

Immunostaining of whole worms

Age synchronized, adult day 1, whole worms were fixed and stained as described in WormBook [48]. Antibodies used include:

rabbit anti-TDP-43 (1:50, Proteintech), rabbit anti-FUS/TLS (1:50, AbCam), and rabbit anti-GABA (1:50, Proteintech).

Fluorescence microscopy

For scoring gaps/breaks from TDP-43 and FUS transgenics, synchronized animals were selected at days 1, 5 and 9 of adulthood for visualization of motor neurons *in vivo*. Animals were immobilized in M9 with 5 *mM* levamisole and mounted on slides with 2% agarose pads. Motor neurons were visualized with a Leica 6000 microscope and a Leica DFC 480 camera. A minimum of 100 animals was scored per treatment over 4–6 trials. The mean and SEM were calculated for each trial and two-tailed t-tests were used for statistical analysis.

Worm lysates

Worms were collected in M9 buffer, washed 3 times with M9 and pellets were placed at −80°C overnight. Pellets were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris pH 7.4, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate)+0.1% protease inhibitors (10 mg/ml leupeptin, 10 mg/ml pepstatin A, 10 mg/ml chymostatin LPC; 1/1000). Pellets were passed through a $27_{1/2}$ G syringe 10 times, sonicated and centrifuged at 16000*g*. Supernatants were collected.

Protein quantification

All supernatants were quantified with the BCA Protein Assay Kit (Thermo Scientific) following the manufacturer instructions.

Protein solubility

For TDP-43 and FUS transgenics soluble/insoluble fractions, worms were lysed in Extraction Buffer (1 M Tris-HCl pH 8, 0.5 M EDTA, 1 M NaCl, 10% NP40+protease inhibitors (LPC; 1/1000)). Pellets were passed through a $27_{1/2}$ G syringe 10 times, sonicated and centrifuged at 100000*g* for 5 min. The soluble supernatant was saved and the remaining pellet was resuspended in extraction buffer, sonicated and centrifuged at 100000g for 5 min. The remaining pellet was resuspended into 100 µl of RIPA buffer, sonicated until the pellet was resuspended in solution and saved.

Immunoblots

Worm RIPA samples (175 µg/well) were resuspended directly in 1× Laemmli sample buffer, migrated in 12.5% or 10% polyacrylamide gels, transferred to nitrocellulose membranes (BioRad) and immunoblotted. Antibodies used: rabbit anti-human-TDP-43 (1:200, Proteintech), rabbit anti-human-FUS/TLS (1:200, AbCam), and mouse anti-actin (1:10000, MP Biomedical). Blots were visualized with peroxidase-conjugated secondary antibodies and ECL Western Blotting Substrate (Thermo Scientific). Densitometry was performed with Photoshop (Adobe).

Statistics

For paralysis and stress-resistance tests, survival curves were generated and compared using the Log-rank (Mantel-Cox) test, and 60–100 animals were tested per genotype and repeated at least three times. For image analysis statistical significance was determined by Student's t-test and the results shown as mean ± standard error. Prism 5 (GraphPad Software) was used for all statistical analyses.

Supporting Information

Table S1 Lifespan analysis for all experiments.
(PDF)

Video S1 mTDP-43 worms in liquid culture at time 0.
(MOV)

Video S2 mTDP-43 worms after 6 hours in liquid culture.
(MOV)

Video S3 mFUS worms in liquid culture at time 0.
(MOV)

Video S4 mFUS worms after 6 hours in liquid culture.
(MOV)

Video S5 wtTDP-43 worms in liquid culture at time 0.
(MOV)

Video S6 wtTDP-43 worms after 6 hours in liquid culture.
(MOV)

Video S7 wtFUS worms in liquid culture at time 0.
(MOV)

Video S8 wtFUS worms after 6 hours in liquid culture.
(MOV)

Video S9 N2 worms in liquid culture at time 0.
(MOV)

Video S10 N2 worms after 6 hours in liquid culture.
(MOV)

Video S11 *unc-47p::GFP* worms in liquid culture at time 0.
(MOV)

Video S12 *unc-47p::GFP* worms after 6 hours in liquid culture.
(MOV)

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Author Contributions

Conceived and designed the experiments: JAP. Performed the experiments: AV AT DA. Analyzed the data: AV JAP. Contributed reagents/materials/analysis tools: GAR PD. Wrote the paper: AV JAP.

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Methylene Blue Protects against TDP-43 and FUS Neuronal Toxicity in *C. elegans* and *D. rerio*

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Abstract

The DNA/RNA-binding proteins TDP-43 and FUS are found in protein aggregates in a growing number of neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS) and related dementia, but little is known about the neurotoxic mechanisms. We have generated *Caenorhabditis elegans* and zebrafish animal models expressing mutant human TDP-43 (A315T or G348C) or FUS (S57Δ or R521H) that reflect certain aspects of ALS including motor neuron degeneration, axonal deficits, and progressive paralysis. To explore the potential of our humanized transgenic *C. elegans* and zebrafish in identifying chemical suppressors of mutant TDP-43 and FUS neuronal toxicity, we tested three compounds with potential neuroprotective properties: lithium chloride, methylene blue and riluzole. We identified methylene blue as a potent suppressor of TDP-43 and FUS toxicity in both our models. Our results indicate that methylene blue can rescue toxic phenotypes associated with mutant TDP-43 and FUS including neuronal dysfunction and oxidative stress.

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Introduction

ALS is a late-onset progressive neurodegenerative disease affecting motor neurons and ultimately resulting in fatal paralysis [1,2]. The majority of cases are sporadic but ~10% of patients have an inherited familial form of the disease. Dominant mutations in SOD1 (copper/zinc superoxide dismutase 1) account for ~20% of familial ALS cases and ~1% of sporadic cases [1]. A recent biochemical approach identified cytosolic aggregates of TDP-43 in ALS and frontotemporal lobar dementia pathological tissue [3]. This breakthrough discovery was quickly followed by the identification of TDP-43 mutations in ALS patients by numerous groups [3–6]. TDP-43 is a multifunctional RNA/DNA binding protein and mutations in the related protein FUS have also been found in ALS patients [7] though the molecular pathology induced by mutant TDP-43 and FUS is not understood. The mislocalization and subsequent aggregation of TDP-43 has been observed in pathological tissue obtained from a number of neurological disorders including frontotemporal lobar dementia, Parkinson's disease, polyglutamine diseases and several myopathies [8]. Similarly, FUS inclusions have been observed in clinically distinct forms of frontotemporal lobar dementia and the polyglutamine diseases [8] suggesting that TDP-43 and FUS may be a common pathogenic factor in neurodegeneration. Furthermore, TDP-43 and FUS interact genetically (though not with SOD1) in zebrafish [9] and *Drosophila* [10] indicating that they may act in a common pathway. In the absence of knowledge concerning the biochemical defects caused by these ALS-related mutations in TDP-43 and FUS, the use of *in vivo* models is currently the most promising approach available to further our understanding of pathogenic mechanisms as well as for therapeutic discovery for ALS.

Indeed a number of chemical and drug screens have been published using *in vivo* models such as *C. elegans* and zebrafish [11–14]. These model organisms offer several advantages over mouse models for cheaper, faster and large-scale initial drug screening and target characterization. For instance, it is possible to rapidly produce large numbers of mutant offspring that can be assayed in liquid culture in multiwell plates and treated with various compounds to determine if disease phenotypes are rescued. Moreover, these organisms have relatively short reproductive cycles, they are easy to manipulate genetically, and their transparency permits visual assessment of developing cells and organs. Also, biochemical pathways are highly conserved between *C. elegans*, zebrafish and humans. We developed novel *in vivo* genetic models of mutant human TDP-43 and FUS in *C. elegans* [15] and zebrafish [9,16,17]. Our models exhibit several aspects of ALS including motor neuron degeneration, axonal deficits and progressive paralysis. The goal of this study was to test the ability of our *in vivo* models to identify neuroprotective compounds and determine their suitability as a platform for pre-clinical drug discovery in ALS. We focused on three compounds with known

neuroprotective properties in an attempt to identify small molecules that might rescue disease phenotypes observed in our models. Here, we show that methylene blue (MB) restores normal motor phenotypes in *C. elegans* and zebrafish ALS models.

Results

Methylene blue rescues mutant TDP-43 and FUS behavioral phenotypes in *C. elegans*

Using *C. elegans* transgenics that express mutant TDP-43 or FUS (TDP-43[A315T] or FUS[S57Δ], referred to herein as mTDP-43 and mFUS respectively) in motor neurons [15] we evaluated the efficacy of these models as drug discovery tools by testing three compounds with known clinically neuroprotective properties: lithium chloride, MB and riluzole [18,19]. The mTDP-43 and mFUS transgenic worms show adult-onset, progressive motility defects leading to paralysis when grown under standard laboratory conditions on solid agar plates over the course of 10 to 12 days [15]. However, worms grown in liquid culture exhibit a swimming behavior that is more vigorous than crawling on plates and accelerates neuronal dysfunction in the TDP-43 and FUS transgenics [15]. As a result, paralysis phenotypes manifest in a matter of hours instead of days. We took advantage of this phenomenon to develop a chemical screening assay to identify compounds that suppress the acute paralysis of mTDP-43 and mFUS transgenic worms grown in liquid culture. With this assay we tested if lithium chloride, MB or riluzole could suppress the paralysis caused by mTDP-43 and mFUS (**Figure 1**). Of the three compounds tested, we observed that MB reduced the rate of paralysis for mTDP-43 and mFUS transgenics with no effect on wild type TDP-43 (wtTDP-43) or wild type FUS (wtFUS) control strains (**Figures 1B, 1E**). Furthermore MB had no significant effect on movement phenotypes for wild type, non-transgenic N2 worms (**Figure S1A**).

To ensure that suppression of paralysis was not an artifact of the liquid culture assay and to confirm that MB retained its rescuing activity in the context of aging we retested it at two doses (6 and 60 μM) for mTDP-43 and mFUS worms grown on plates and observed a reduction in the rates of paralysis for treated animals compared to untreated controls (**Figures 2A, B**). The paralysis phenotype likely results from impaired synaptic transmission at the neuromuscular junction as shown by the hypersensitivity of the mTDP-43 worms to the acetylcholine esterase inhibitor aldicarb. mTDP-43 animals treated with MB showed reduced sensitivity to aldicarb, matching the response from control strains, suggesting that MB restores synaptic function in animals expressing mutant proteins (**Figure 2C**). Transgenic *C. elegans* expressing ALS-related mutations mTDP-43 or mFUS in motor neurons also show age-dependent degeneration most frequently observed as gaps or breaks along neuronal processes [15]. These neurodegenerative phenotypes were significantly reduced by treatment with MB (**Figures 2D, E, F**) and did not change mTDP-43 or mFUS transgene expression (**Figures 2G, H**).

Methylene blue rescues motor phenotypes in mutant TDP-43 and FUS zebrafish

To test if MB had protective effects beyond *C. elegans* we turned to zebrafish. First, as in worms, we observed that MB had no effect on the movement phenotypes of wild type non-transgenic fish (**Figure S1B, C, D, E**). Zebrafish expressing mTDP-43[G348C] or mFUS[R521H] have impaired swimming as assessed by their ability to produce a touch-evoked escape response (TEER) [9,16]. mTDP-43 fish showed a greatly reduced TEER compared to non-transgenic or wtTDP-43 fish (**Figure 3A**). mTDP-43 fish treated with 60 μM MB showed improved swimming response including swim duration, distance swam and maximum swim velocity (**Figures 3A, B, C, D**). Zebrafish expressing mFUS also show greatly reduced swimming activity compared to wild type or wtFUS fish and the swimming phenotype of mFUS fish was greatly improved when treated with 60 μM MB (**Figures 3E, F, G, H**). Besides behavioral defects, immunohistochemical analyses show that transgenic zebrafish expressing mTDP-43 or mFUS also displayed abnormally shortened and branched motor neuron axonal processes as observed by the unbranched axonal length (UAL) quantification [9,16] and this phenotype was rescued by incubation with either 30 or 60 μM MB (**Figures 4A, B**). These results demonstrate that MB can significantly reduce the motor neuron phenotypes elicited by expression of mTDP-43 and mFUS both in *C. elegans* and zebrafish genetic models of disease.

Methylene blue protects against oxidative stress in *C. elegans* and zebrafish

Since we observed that MB rescued paralysis in transgenic models of mTDP-43 or mFUS, we sought to further examine the protective effects of MB in an aging and stress context. First, MB treatment had no effect on the lifespan of wild type N2 worms suggesting that its cellular protection mechanisms are not due to non-specific effects from extended longevity (**Figure 5A, Table S1**). To test for protective effects against environmental stress we tested wild type N2 worms for their ability to withstand lethal exposure to thermal, hyperosmotic or oxidative stresses. We observed that MB offered no protection to worms subjected to elevated temperature or hyperosmotic stress from treatment with NaCl as their survival rate was indistinguishable from untreated control animals (**Figures 5B, C**). Juglone is a natural aromatic compound found in the black walnut tree that induces high levels of oxidative stress within cells [20]. Juglone is highly toxic to wild type N2 worms and causes complete mortality after approximately 4 hours in our assay. We observed that MB provided significant protection against oxidative stress since wild type N2 worms were resistant to juglone in a dose dependent manner (**Figure 5D**). These data suggest that MB is specific in its cell protection capabilities and helps overcome oxidative stress conditions in *C. elegans*.

Since we showed that MB confers protection to wild type N2 worms under oxidative stress in a dose dependent manner we hypothesized that MB may help reduce oxidative damage in mTDP-43 worms. To test this hypothesis we stained our TDP-43 transgenic strain with dihydrofluorescein diacetate (DHF), a compound known to fluoresce when exposed to intracellular peroxides associated with oxidative stress [21]. We observed no DHF signal from wtTDP-43 transgenics but strong fluorescence from mTDP-43 worms (**Figure 6A**). The fluorescence observed in the mTDP-43 transgenics was reduced when treated with 60 μM MB (**Figure 6A**). We observed a similar effect with our FUS transgenics, with no DHF signal from wtFUS animals, but strong fluorescence from mFUS worms that was reduced upon MB treatment (**Figure 6B**). Extending our findings we examined oxidative stress with DHF in mTDP-43 and mFUS fish. Similar to worms, we observed a strong fluorescent signal in mTDP-43 fish compared to non-transgenic or wtTDP-43 fish and that this signal was reduced by treatment with MB (**Figures 6C, D**). MB also reduced the fluorescent signal in mFUS fish stained with DHF (**Figure 6E**). These data suggest that MB reduces the general level of oxidative status generated by the expression of mutant proteins *in vivo*.

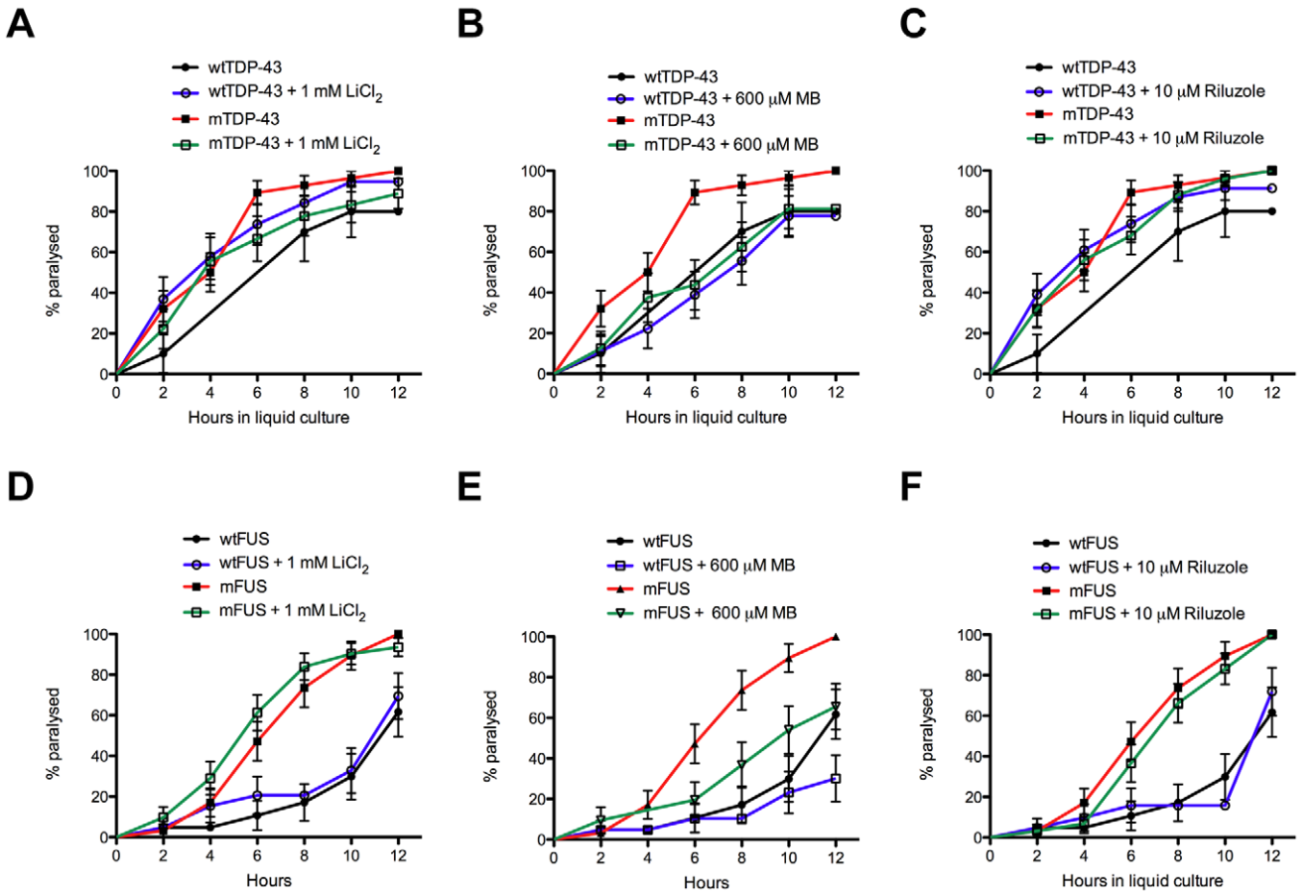

Figure 1. Methylene blue suppresses mTDP-43 and mFUS associated paralysis in *C. elegans*. We screened for suppression of TDP-43 (A–C) and FUS (B–D) induced paralysis in liquid culture by lithium chloride ($LiCl_2$), methylene blue (MB) or riluzole. MB significantly reduced the rate of paralysis in (B) mTDP-43 and (D) mFUS transgenics compared to untreated mutant transgenic worms ($P<0.05$) with no effect on wild type transgenic controls.
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Reduced neuroprotection from late administration of methylene blue

In the previous experiments worms and fish were treated with MB from hatching. We tested whether the timing of treatment had an effect on the magnitude of neuroprotection by growing mTDP-43 worms on normal plates and transferring them at day 5 of adulthood to plates supplemented with MB. We observed that late administration of MB reduced paralysis with approximately 55% of treated animals becoming paralysed at day 12 of adulthood compared to a paralysis rate of approximately 80% for untreated animals (**Figure 7**). However the extent of rescue by late MB administration was far less than the approximate 10% paralysis rate observed for mTDP-43 animals grown on MB plates from hatching (**Figure 2A**). These data suggest that early administration of MB is more effective at reducing mTDP-43 toxicity than intervention in older animals.

Discussion

In this study we demonstrated that our *C. elegans* and zebrafish ALS models can be used to identify neuroprotective molecules which represents the first *in vivo* chemical genetic screening platform for ALS. With this platform we discovered that MB is a potent suppressor of mTDP-43 and mFUS motor neuron toxicity *in vivo*. In both worms and fish MB corrected motor deficits and reduced the level of oxidative stress associated with the expression of mutant proteins.

MB is a pleiotropic molecule with a long and varied history of medical use [18] but in the context of neurodegeneration MB has been reported to prevent amyloid-β and tau aggregation *in vitro* [22,23]. A previous study also showed that the treatment of cells with MB inhibited the formation of TDP-43 aggregates [24] suggesting this compound might be appropriate for the treatment of ALS and other dementias. The efficacy of MB as a neuroprotective compound has been examined in Alzheimer's disease and ALS models where in some studies it is protective while in others it has no effect [24–28]. We decided to include this compound in our assay from which we identified MB as a potent suppressor of mTDP-43 and mFUS toxicity in both *C. elegans* and zebrafish. However, our data do not agree with a recent study examining the effects of MB in a TDP-43 mouse model [26]. Mutant TDP-43[G348C] mice treated with MB showed no improvement in motor phenotypes as determined by the rotarod assay. Furthermore no difference in the cytoplasmic localization of TDP-43 was observed in treated mice.

Worms and fish live in aqueous media and a simple explanation for their greater susceptibility may be that they are more permeable to MB. We further hypothesize that the differences in MB efficacy might also be due to variations in timing for delivery of the compound. Specifically, our worms and fish were treated

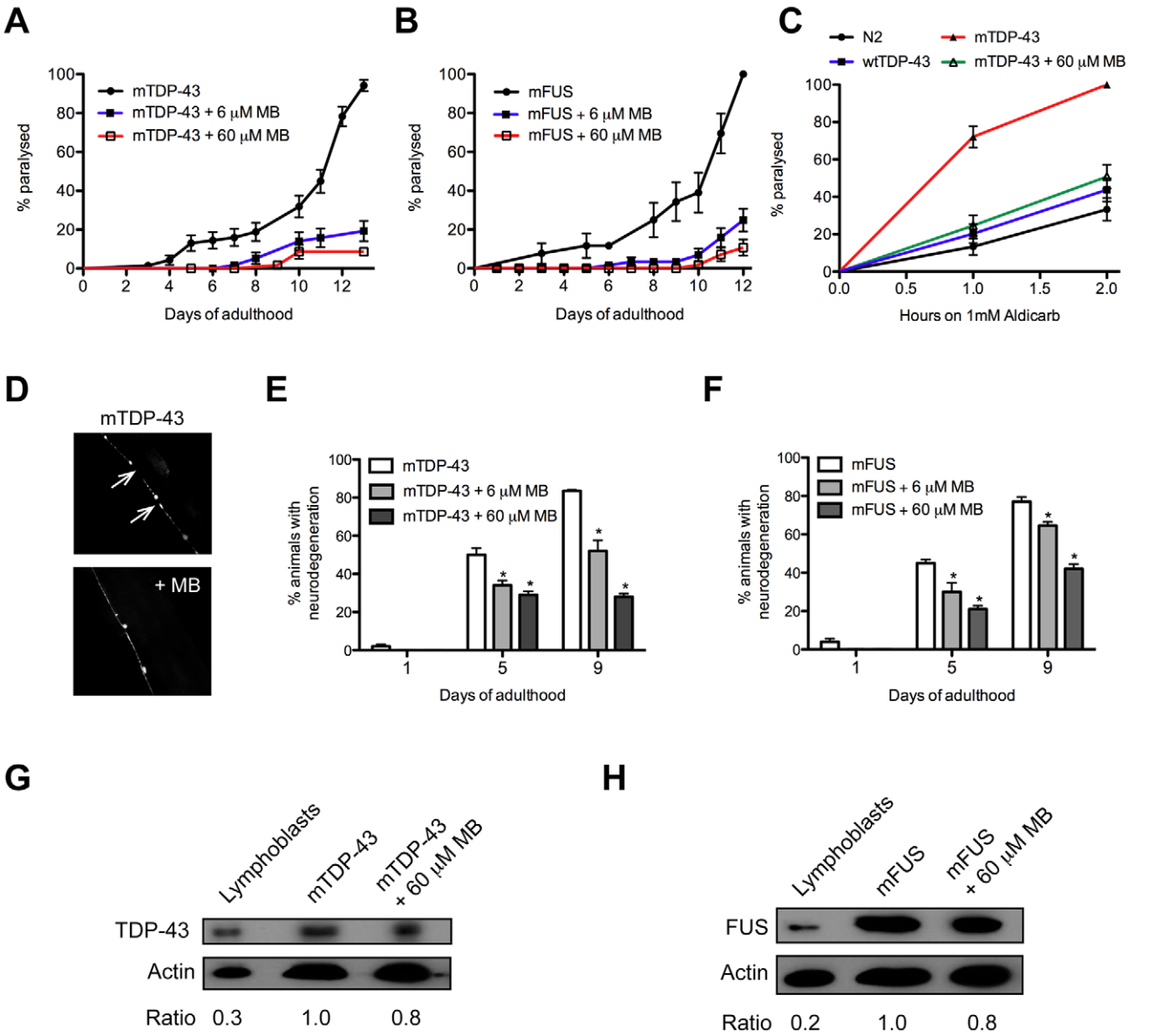

Figure 2. Methylene blue reduces TDP-43 and FUS neuronal toxicity. mTDP-43 and mFUS transgenics were grown on plates and assayed for various phenotypes. (**A**) MB reduced mTDP-43 induced paralysis in worms at two doses compared to untreated controls ($P<0.001$). (**B**) MB at two doses reduced mFUS induced paralysis in worms compared to untreated controls ($P<0.001$). (**C**) Aldicarb induced paralysis for mTDP-43 worms is significantly higher for mTDP-43 worms compared to non-transgenic N2 worms or transgenic wtTDP-43 controls ($P<0.001$). MB reduced aldicarb induced paralysis of mTDP-43 worms back to non-transgenic N2 and wtTDP-43 levels. (**D**) Representative photos of motor neuron degeneration phenotypes observed in mTDP-43 transgenic worms. Similar phenotypes were observed for mFUS transgenics. Degeneration is most frequently seen as gaps (white arrows) along neuronal processes. MB reduced the age-dependent degeneration of motor neurons in (**E**) mTDP-43 and (**F**) mFUS transgenic worms (*$P<0.001$ compared to untreated transgenics). MB did not affect the expression of mutant proteins in (**G**) mTDP-43 or (**H**) mFUS strains as determined by western blotting of protein extracts from transgenic worms grown with or without MB. Immunoblotting of human lymphoblasts was used as a size control.
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with MB from hatching whereas the TDP-43 mice were treated at 6 months. To confirm this hypothesis we treated mTDP-43 worms with MB at day 5 of adulthood and observed that late administration of the compound was significantly less effective at reducing paralysis. Thus, perhaps earlier (pre-clinical) treatment with MB may have greater effects in mouse models for ALS. Additionally there may be differences between the models since our worm and fish models capture a clinical aspect of ALS, namely progressive paralysis in animals expressing mTDP-43 that is absent from the TDP-43 mouse model.

Aging is a risk factor common to a number of neurodegenerative disorders including ALS, and oxidative stress is suspected to play a key role in the development of the disease by contributing to aging [29,30]. Indeed, interactions between genetic, environmental, and age-dependent risk factors have been hypothesized to trigger disease onset [31]. Consequently, we investigated the impact of MB treatment focusing on aging and stress response. Our *C. elegans* data are in agreement with the survival data from the mouse studies where we observed no effect on lifespan in MB treated worms even though there was a positive effect on multiple

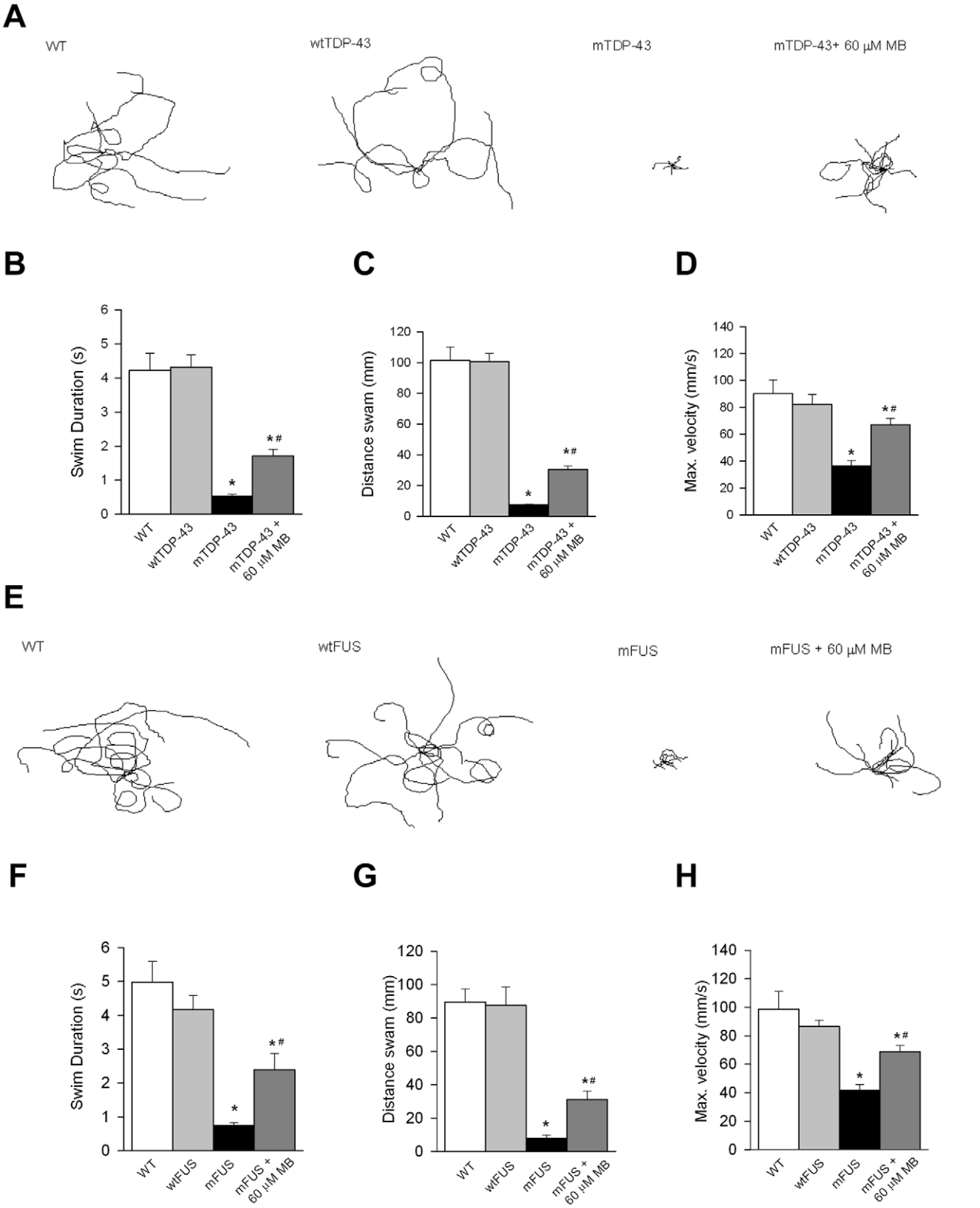

Figure 3. Methylene blue reduces motor deficits in zebrafish expressing mutant TDP-43 or FUS. (**A**) Representative traces of TEER phenotypes in wild type (WT), wtTDP-43, mTDP-43 and mTDP-43+MB. MB improved the swim duration (**B**), distance swam (**C**) and maximum swimming velocity (**D**) of mTDP-43 fish. (**E**) Representative traces of TEER phenotypes in WT, wtFUS, mFUS and mFUS+MB. Application of MB led to a significant improvement in the swim duration (**F**), distance swam (**G**) and maximum swimming velocity (H) of mTDP-43 fish. * denotes significant difference from WT, $P<0.001$; # significantly different from mutant fish $P<0.05$.
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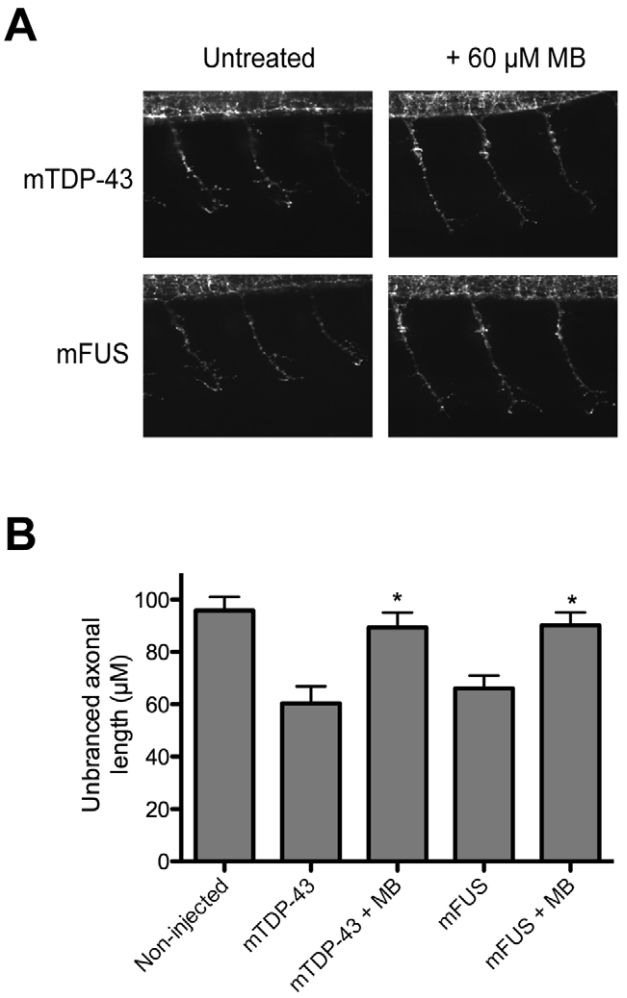

Figure 4. Methylene blue reduces axon defects in zebrafish expressing mutant TDP-43 or FUS. (**A**) Appearance of motor neurons in mTDP-43 and mFUS transgenic zebrafish with and without MB treatment. (**B**) MB reduced the unbranched axon length phenotype of motor neurons in mTDP-43 and mFUS transgenic zebrafish (*P<0.01 compared to untreated transgenics).
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phenotypes associated with mTDP-43 or mFUS. Thus, at least in simple systems lifespan effects can be uncoupled from neuroprotection but it remains to be seen if the same is true for mouse models of neurodegeneration.

In our previous work we showed that our TDP-43 and FUS transgenic *C. elegans* models exhibited no difference in lifespan compared to non-transgenic worms [15]. Thus, the paralysis phenotypes observed in our models specifically reflect the consequences of the expression of TDP-43 and FUS in motor neurons and are not due to secondary effects from general sickness and reduced lifespan. Therefore, it may be difficult to detect significant improvement on motor function or reflex phenotypes after MB treatment in mice showing generalized defects instead of treating problems resulting from TDP-43 or FUS proteotoxicity alone.

Finally, the TDP-43 mouse study did not examine the effects of MB on synaptic function or oxidative stress where we see clear effects in the worm and zebrafish models. MB can interact with nitric oxide synthase and also has an antioxidant potential by decreasing the generation of reactive oxygen species [32]. Using *C. elegans* we showed that MB specifically decreased the sensitivity of wild type worms to oxidative stress. We also investigated the impact of MB treatment in the formation of reactive oxygen species in both *C. elegans* and *D. rerio* and have observed a significant reduction in the generation of reactive oxygen species. Consistent with the literature [33], our data suggest that MB counteracts oxidative stress to provide protection against proteotoxicity in both our *in vivo* models. Synaptic function was also restored after treatment with MB in transgenic mTDP-43 worms suggesting that this compound might also have an effect on synaptic transmission.

In summary, we present novel *in vivo* chemical genetic screening assays that may be useful for ALS drug discovery. Using two genetic models for ALS we report here that MB acts through reduction of oxidative stress and also restoration of normal synaptic function in genetic models of ALS. In addition, an important issue here is that in simple systems like *C. elegans*, lifespan effects can be uncoupled from neuroprotection. The next step will be to unravel MB's exact target and mechanism of action to develop compounds with more specific activities and also to capitalize on the strength of our assays to screen additional compounds as potential therapeutics in ALS.

Materials and Methods

C. elegans experiments

***C. elegans* strains.** Strains used in this study include: N2, *gas-1(fc21), let-60(ga89), oxIs12[unc-47p::GFP;lin-15(+)], xqIs98[unc-47::FUS[S57Δ];unc-119(+)], xqIs132[unc-47::TDP-43-WT;unc-119(+)], xqIs133[unc-47::TDP-43[A315T];unc-119(+)]* and *xqIs173[unc-47::FUS-WT;unc-119(+)]*.

***C. elegans* liquid culture assay.** Young adult TDP-43 or FUS transgenic worms were distributed in 96-wells plate (20 μl per well; 20–30 worms per well), containing DMSO or test compounds and incubated for up to 12 hours at 20°C on a shaker. Compounds and final concentrations tested were 1 mM lithium chloride, 600 μM methylene blue, and 10 μM riluzole. The motility test was assessed by microscopy every 2 hours. Compounds were purchased from Sigma-Aldrich (St-Louis, MO).

***C. elegans* drug testing on plates.** Worms were grown on standard NGM plates with or without compounds. For worms expressing mFUS or mTDP-43, animals were counted as paralyzed if they failed to move upon prodding with a worm pick. Worms were scored as dead if they were immotile, showed no pharyngeal pumping and failed to move their head after being prodded in the nose. The final concentrations of methylene blue tested in plates either 6 or 60 μM.

Fluorescence microscopy. For scoring axons from transgenic mFUS and mTDP-43 worms, synchronized animals were selected at days 1, 5 and 9 of adulthood for visualization of motor neurons *in vivo* with the *unc-47p*::GFP transgenic reporter. Animals were immobilized in M9 with 5 mM levamisole and mounted on slides with 2% agarose pads. Motor neurons were visualized with a Leica CTR 6000 and a Leica DFC 480 camera. A minimum of 100 animals was scored per treatment over 4–6 trials. Animals showing gaps or breaks along motor neuron processes were scored as positive for the degeneration phenotype. The mean and SEM were calculated for each trial and two-tailed *t*-tests were used for statistical analysis. For visualization of fluorescence after treatment with dihydrofluorescein diacetate, L4 animals were grown on NGM plates or NGM plates with methylene blue and examined for fluorescence with the Leica system described above.

Lifespan assays. Worms were grown on NGM or NGM+60 μM methylene blue and transferred on NGM-FUDR or NGM-FUDR+60 μM methylene blue. 20 animals/plate by

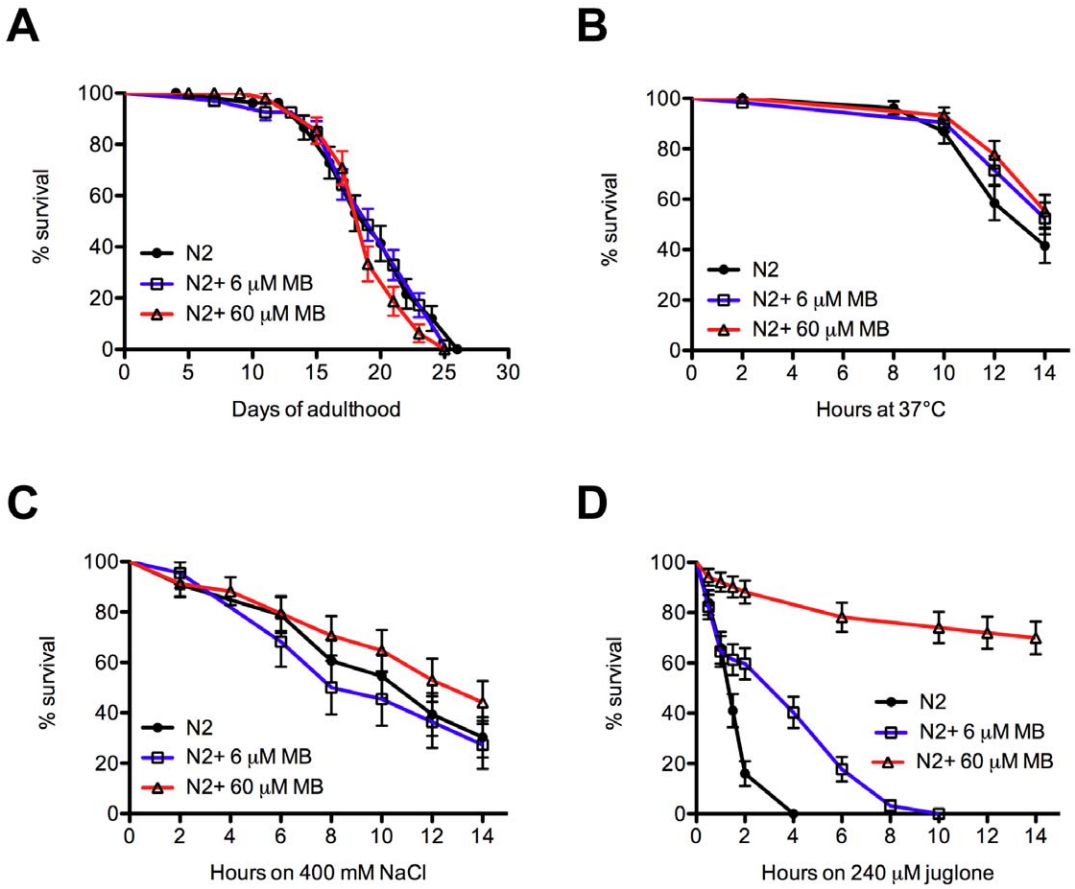

Figure 5. Methylene blue protects against oxidative stress in *C. elegans*. (**A**) N2 worms grown on plates with MB had lifespans indistinguishable from untreated worms (see also Table S1). (**B**) Worms grown on MB and subjected to thermal stress showed similar survival rates compared to untreated N2 worms. (**C**) N2 worms treated with MB showed similar rates of survival compared to untreated worms when subjected to hyperosmolarity. (**D**) MB had a dose-dependent protective effect on N2 worms against oxidative stress and mortality when grown on plates containing juglone ($P<0.001$ for MB treated N2 worms compared to untreated worms).
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triplicates were tested at 20°C from adult day 1 until death. Worms were declared dead if they were immotile and did not respond to tactile or heat stimulus.

Stress assays. For oxidative stress tests, worms were grown on NGM or NGM+60 μM methylene blue and transferred to NGM plates +240 μM juglone at adult day 1. For thermal resistance worms were grown on NGM or NGM 60 μM methylene blue and put at 37°C at adult day 1. For osmotic resistance worms were grown on NGM or NGM+60 μM methylene blue and put on 400 mM NaCl plates at adult day 1. For all assays, worms were evaluated for survival every 30 min for the first 2 hours and every 2 hours after up to 14 hours. Nematodes were scored as dead if they were immotile and unable to move in response to heat or tactile stimuli. For all tests worms, 20 animals/plate by triplicates were scored.

Dihydrofluorescein diacetate assay. For visualization of oxidative damage in the transgenic strains the worms were incubated on a slide for 30 min with 5 μM dihydrofluorescein diacetate dye and then washed with 1× PBS three times. After the slide was fixed, fluorescence was observed with the Leica system described above.

Worm lysates. Worms were collected in M9 buffer, washed 3 times with M9 and pellets were placed at −80°C overnight. Pellets were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris pH 7.4, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate)+protease inhibitors (10 mg/ml leupeptin, 10 mg/ml pepstatin A, 10 mg/ml chymostatin LPC;1/1000). Pellets were passed through a $27_{1/2}$ G syringe 10 times, sonicated and centrifuged at 16,000× *g*. Supernatants were collected.

Immunoblot. Worm RIPA samples (175 μg/well), lymphoblast cell RIPA samples (15 μg/well) were resuspended directly in 1× Laemmli sample buffer, migrated in 12.5% polyacrylamide gels, transferred to nitrocellulose membranes (BioRad) and immunoblotted. Antibodies used: rabbit anti-TDP-43 (1:200; Proteintech), rabbit anti-FUS/TLS (1:200; AbCam), and mouse anti-actin (1:10000 for worms, MP Biomedicals). Blots were visualized with peroxidase-conjugated secondary antibodies and ECL Western Blotting Substrate (Thermo Scientific).

Statistical analysis. For paralysis and stress-resistance tests, survival curves were generated and compared using the Log-rank (Mantel-Cox) test, and a 60–100 animals were tested per genotype and repeated at least three times.

Zebrafish experiments

Zebrafish maintenance. Zebrafish (*Danio rerio*) embryos were raised at 28.5°C, and collected and staged using standard methods [34]. The Comité de Déontologie de l'Expérimentation sur les Animaux (CDEA), the local animal care committee at the Université de Montréal, having received the protocol relevant to

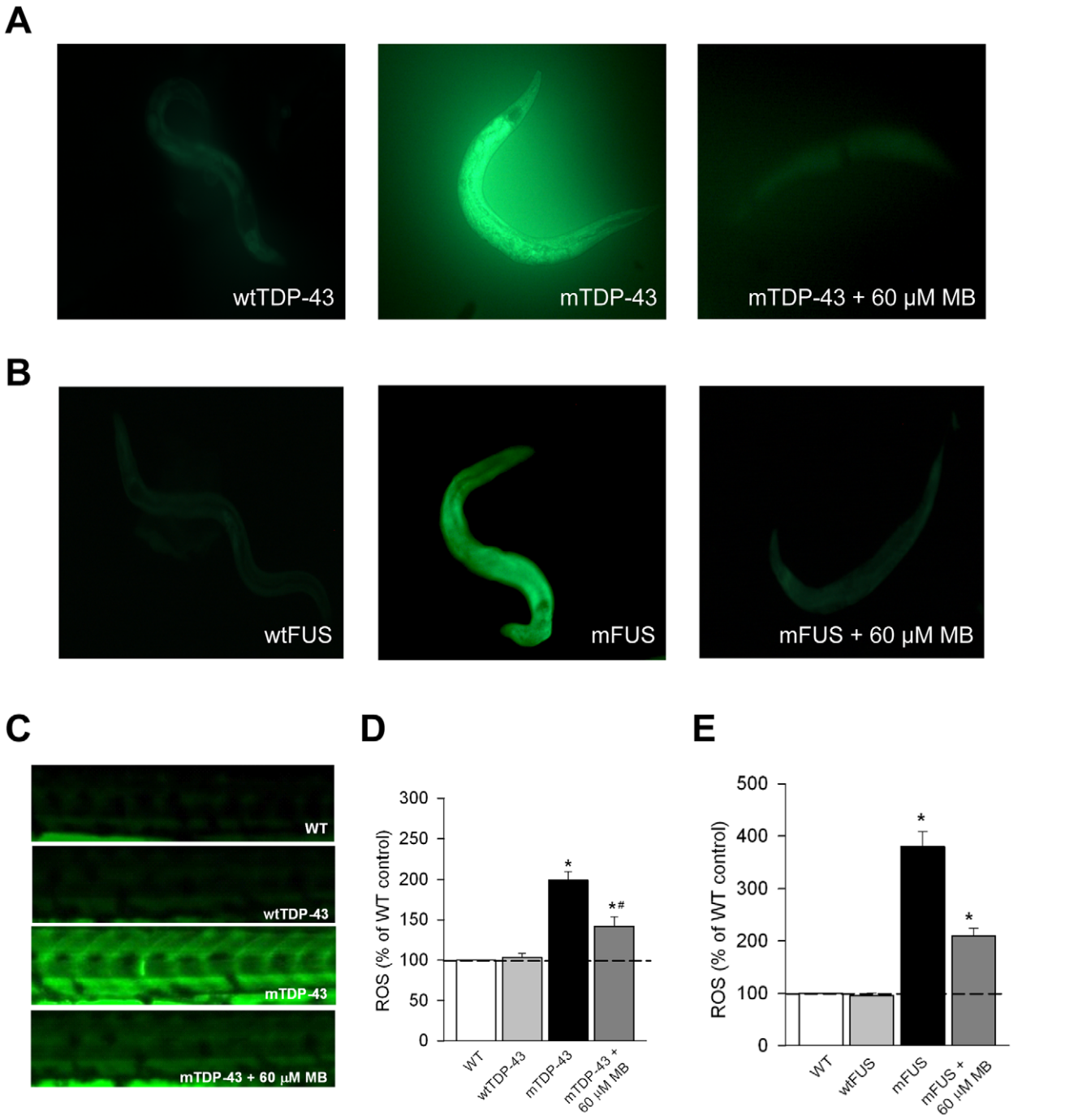

Figure 6. Methylene blue reduces oxidative stress in *C. elegans* and zebrafish transgenics. Oxidative stress was measured in transgenic worms and zebrafish with the dihydrofluorescein diacetate (DHF) that fluoresces when exposed to intracellular peroxide. (A) mTDP-43 worms, but not wtTDP-43 transgenics have a higher level of oxidative stress when stained with DHF. mTDP-43 worms treated with MB and then stained with DHF show a remarkable reduction in fluorescence. (B) wtFUS worms show no fluorescence when stained with DHF compared to mFUS worms. mFUS worms treated with MB and then stained with DHF showed reduced fluorescence. (**C**) Wild type (WT) zebrafish and zebrafish expressing wtTDP-43 show very low levels of fluorescence when stained with DHF compared to mTDP-43 fish. Treatment with MB reduced fluorescence in DHF stained fish. (**D**) Quantification of fluorescence of DHF stained fish shows that MB treatment significantly reduced fluorescence in mTDP-43 fish (*$P<0.001$, *#$P<0.01$). (**E**) MB significantly reduced fluorescence in DHF stained mFUS zebrafish (*$P<0.001$).
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this project relating to animal care and treatment, certified that the care and treatment of animals was in accordance with the guidelines and principles of the Canadian Council on Animal Care. Further, all matters arising from this proposal that related to animal care and treatment, and all experimental procedures proposed for use with animals were reviewed and approved by the CDEA before they were initiated or undertaken. This review process was ongoing on a regular basis during the entire period

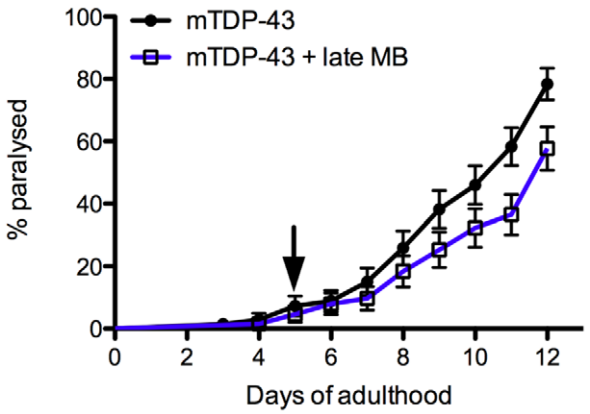

Figure 7. Diminished neuroprotection from late administration of methylene blue. mTDP-43 worms grown on normal plates and switched to plates supplemented with 60 μM MB at day 5 (indicated by the arrow) of adulthood (late MB) showed a modest but significant reduction in paralysis compared to untreated worms ($P<0.05$).
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that the research was being undertaken. Zebrafish embryos (no adults were used) are insentient to pain. Fish embryos were incubated overnight in each compound and examined the next day and then disposed. Zebrafish embryos were used over a two-day period then terminated.

In-vitro mRNA synthesis and embryo microinjection. Human FUS wild type and mutant [R521H], human TDP-43 wild type and mutant [G348C] mRNAs were transcribed from NotI-linearized pCS2+ using SP6 polymerase with the mMESSAGE Machine Kit (Ambion). This was followed by a phenol:chloroform extraction and isopropanol precipitation, and diluted in nuclease-free water (Ambion). The mRNAs were diluted in nuclease free water (Ambion) with 0.05% Fast Green vital dye (Sigma-Aldrich) at a concentration of 60 ng/μl (FUS), 25 ng/μl (TDP-43) and were pulse-injected into 1–2 cell stage embryos using a Picospritzer III pressure ejector.

Chemical treatments. Transient transgenics for TDP-43 [G348C] and FUS [R521H] embryos at 24 hpf were placed in individual wells in a 24 well plate and were treated overnight with methylene blue diluted in Evans solution (in mM): 134 NaCl, 2.9 KCl, 2.1 $CaCl_2$, 1.2 $MgCl_2$, 10 HEPES, 10 glucose, pH 7.8, 290 mOsm, with 0.1% DMSO. Behavioural touch responses were then assessed at 52–56 hpf as described in the following section.

Touch-evoked escape response. Zebrafish larvae were touched lightly at the level of the tail with a pair of blunt forceps and their locomotor behavior was recorded with a Grasshopper 2 Camera (Point Grey Research) at 30 Hz. The movies were then analyzed using the manual tracking plugin of ImageJ 1.45r software (NIH) and the swim duration, swim distance and maximum swim velocity of the fish were calculated.

Unbranched axonal length measurements. For immunohistochemical analysis of axonal projections of motor neurons, monoclonal antibody anti-SV2 (Developmental Studies Hybridoma) were used to assess the motor neuron morphology at 48 and 72 hpf. Fluorescent images of fixed embryos were taken using a Quorum Technologies spinning-disk confocal microscope mounted on an upright Olympus BX61W1 fluorescence microscope equipped with an Hamamatsu ORCA-ER camera. Image acquisition was performed with Volocity software (PerkinElmer). As previously described [16], axonal projections from primary and secondary motor neurons at a defined location in the inter somitic segments were determined. Analysis of Z-stacks by confocal microscopy was performed in three to four axonal projections per animal. The axonal length to the first branching (UAL) was determined by tracing the labeled axon from the spinal cord to the point where it branches using ImageJ (NIH). These values were averaged for each of the animal analyzed (10–30 zebrafish per condition) for the various conditions in our study.

Reactive Oxygen Species measurements. For *in vivo* detection of reactive oxygen species, live 2 day old embryos (2 dpf) were incubated in 5 μM 2′,7′-dichlorofluorescein diacetate (Sigma-Aldrich) for 20 minutes at 28.5°C and washed three times for 5 min with embryo media. Fluorescence was observed under a 488 nm wavelength excitation. The generation of reactive oxygen species in the larvae exposed to the chemicals was also quantitatively assessed as described elsewhere [35]. Briefly, 15 embryos were washed with cold Phosphate Buffer solution (PBS; pH 7.4) twice and then homogenized in cold buffer (0.32 mM of sucrose, 20 mM of HEPES, 1 mM of $MgCl_2$, and 0.5 mM of phenylmethyl sulfonylfluoride (PMSF), pH 7.4). The homogenate was centrifuged at 15,000× g at 4°C for 20 min, and the supernatant was transferred to new tubes for further experimentation. Twenty microliters of the homogenate was added to a 96-well plate and incubated at room temperature for 5 min, after which 100 μl of PBS (pH 7.4) and 8.3 μl of DFH stock solution (10 mg/ml) were added to each well. The plate was incubated at 37°C for 30 minutes. The fluorescence intensity was measured using a microplate reader (SpectraMax M2, Molecular Device, Union City, CA, USA) with excitation and emission at 485 and 530 nm, respectively. The reactive oxygen species concentration was expressed as arbitrary emission units per mg protein.

Statistical analysis. All data values are given as means ± SEM. Significance was determined using one-way ANOVAs and Fisher LSD tests for normally distributed and equal variance data, Kruskal–Wallis ANOVA and Dunn's method of comparison were used for non-normal distributions.

Supporting Information

Figure S1 Methylene blue has no effect on wild type motility phenotypes in worms or zebrafish. (**A**) MB had no significant effect on the motility phenotype of wild type (WT) non-transgenic N2 worms. (**B**) Representative traces of TEER phenotypes in WT zebrafish with and without MB treatment. MB did not affect the swim duration (**C**), distance swam (**D**) or maximum swimming velocity (**E**) of WT zebrafish.
(TIF)

Table S1 Lifespan analysis for all experiments. Related to Figure 5A. Animals that died prematurely (ruptured, internal hatching) or were lost (crawled off the plate) were censored at the time of scoring. All control and experimental animals were scored and transferred to new plates at the same time. ns: not significant.
(PDF)

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Author Contributions

Conceived and designed the experiments: EK PD JAP MT. Performed the experiments: AV SAP CM EK SC MT. Analyzed the data: AV SAP EK JAP MT. Wrote the paper: AV SAP PD EK JAP MT.

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